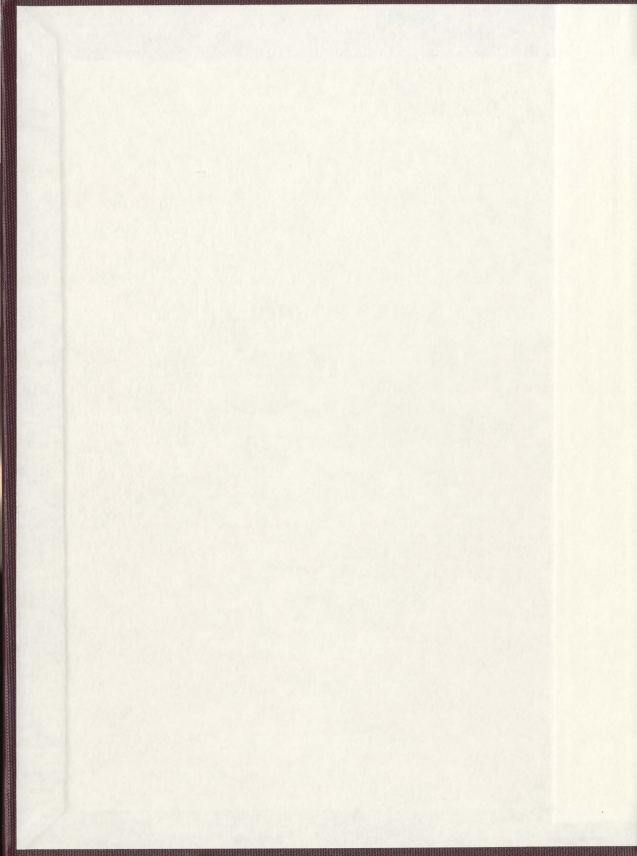


INTERACTION OF SERUM LIPIDS WITH LUNG
SURFACTANT AS MODELS OF ACUTE RESPIRATORY
DISTRESS SYNDROME (ARDS)

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Interaction of Serum Lipids with Lung Surfactant as Models of Acute Respiratory Distress Syndrome (ARDS)

by

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Abstract

Lung surfactant (LS) is a lipid-protein mixture secreted by type II alveolar cells to the air-water interface of the alveoli. LS stabilizes the alveoli and prevents lung collapse during respiration. This occurs by surfactant forming a surface active film at the interface to lower the surface tension (γ) to very low values (~ 1 mN/m) at end expiration. Under certain conditions, LS can become dysfunctional. This is seen during acute respiratory distress syndrome (ARDS) and lung injury, among other lung diseases. During ARDS, serum components, possibly soluble serum proteins and lipids, or a combination of both, can leak into the alveolar space and thereby inactivate LS. Previously it was found in our laboratory that serum, as a whole entity, was two-hundred times more potent an inhibitor than its individual protein components. This could suggest that there are possibly other components of serum than soluble proteins which are potent inactivators of LS.

Interactions of a bovine lipid extract surfactant (BLES) with varying amounts of whole fetal calf serum (FCS), cholesterol, and low density lipoprotein (LDL) were studied using several biophysical techniques to examine serum lipid components and their effects on the structure and function of BLES in films and bilayers. BLES contains all lipids and hydrophobic proteins of LS except neutral lipids (like cholesterol) and the hydrophilic surfactant proteins A and D. This allowed us to study the effects of normal and excess cholesterol, lipoproteins and other serum lipidic materials on this surfactant system. Mass spectrometry and lipid Iatroscan showed LDL and serum lipid fraction to contain significant amounts of cholesterol-ester and lipids not found in BLES.

From our adsorption studies both serum and low density lipoprotein (LDL) hindered the ability of BLES bilayer dispersions to rapidly form films at the air-water interface.

However, low physiological amounts (5-10%) of cholesterol did not disturb the rate of adsorption of BLES. Langmuir-Blodgett films were studied using a Langmuir-Wilhelmy balance to study surface activity and structure of such adsorbed films. All serum lipid components except physiological concentrations of cholesterol (5-10% wt) significantly decreased the surface activity of the films.

Images of these films obtained by atomic force microscopy (AFM), suggested that normal amounts of cholesterol formed gel-like condensed domains until compressed to a low γ . This domain formation was abolished at higher cholesterol concentrations. Whole serum, high cholesterol and LDL prevented BLES domain formation as well as γ reduction and in fact produced non-gel domains. Bilayer dispersions of such samples (used to form the adsorbed films), using Raman spectroscopy, suggested that serum components altered the bond vibrational modes of the fatty acyl chains (C-H, C-C) of BLES phospholipids, compared to serum proteins alone. The bilayer studies suggested that serum lipids components affected the fluidity and lipid packing of BLES and thereby possibly altered molecular arrangements and packing of films formed from such bilayer. The study suggests a molecular model of serum lipid component as an inactivator of LS in ARDS.

The monolayer and bilayer studies suggested that these lipid components are far more potent inhibitors of LS than their protein counterparts and are able to disturb lipid packing and change structure and surface activity of LS.

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List of Abbreviations and Symbols

AECC	American European consensus conference
AFM	atomic force microscopy/microscope
ALI	acute lung injury
APO-B	apo lipoprotein B
ARDS	acute respiratory distress syndrome
BLES	bovine lipid extract surfactant
BSA	bovine serum albumin
CCD	charge coupled device
CRP	C-reactive protein
ddH ₂ O	double distilled water
DPPC	dipalmitoylphosphatidylcholine
DPPG	dipalmitoylphosphatidylglycerol
DSC	differential scanning calorimetry
Fbg	fibrinogen
FCS	fetal calf serum
FTIR	Fourier transform infrared spectroscopy
GUV	giant unilamellar vesicles
γ	surface tension
HDL	high density lipoprotein
LB	lamellar bodies
LBPA	lyso-bis-phosphatidic acid

LDL	low density lipoproteins
LPC	lysophosphatidylcholine
LS	lung surfactant
mg/ml	milligram(s) per milliliter
mN/m	milliNewton(s) per meter
m/z	mass over charge
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MS	mass sepectrometry
μl	microliter(s)
OH	hydroxyl group
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PL	phosholipids
PS	phosphatidylserine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SM	sphingomyelin
SP	surfactant proteins
SP-A	surfactant protein A
SP-B	surfactant protein B
SP-C	surfactant protein C

SP-D	surfactant protein D
TM	tubular myelin



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Chapter 1: Introduction

1.1 Lung Surfactant (LS)

The prevention of lung collapse is vital to allow the lungs to properly function, especially during expiration. For the lungs to function normally, a lipid-protein material called lung surfactant (LS) lines the air-aqueous interface (hypophase) of the alveoli. The alveoli are flaccid sacs in the lung that carry out gas exchange and also produce surfactant. Surfactant is secreted by alveolar Type II cells as bilayer vesicles, called Lamellar Bodies (LB), which transform to form Tubular Myelin (TM). The TM are lipid-protein, cross-hatched structures (Lumb, 1989; Nag, 2005), which are the largest surface-active, aggregate fraction of LS. TM becomes fully functional when it is adsorbed to the air-water interface of alveoli and forms a surface layer of films. Lung surfactant has to be fluid enough to adsorb rapidly but rigid enough to lower surface tension (γ) when the adsorbed film undergoes compression (Clements, 1977; Goerke, 1974; King & Clements, 1972; Possmayer *et al.*, 2010).

LS layers or films formed at the alveolar hypophase function to stabilize the lung by lowering γ close to near 1 mN/m values during compression. LS can adsorb rapidly to an air-water interface to reach equilibrium γ of 25 mN/m *in vitro*. Surface tension is produced when there is a greater attractive force between water molecules in bulk than that of air at the air-aqueous interface. LS serves to lower γ by interacting with the intermolecular forces of attraction of water molecules in the surface and therefore reducing γ of that interface. It is this function of LS that shows that it is surface active and fully functional (Possmayer *et al.*, 2010).

Various lung diseases and injuries may cause LS to function improperly. This is evident in cases of disease, such as Acute Respiratory Disease Syndrome (ARDS) and Acute Lung Injury (ALI), where the normal respiratory processes of the lung are compromised and LS is no longer able to function properly. **Figure 1** compares a normal lung to a lung in ALI (Bailey & Veldhuizen, 2005).

1.2 Composition of LS

In most mammalian species, LS is comprised of approximately 90% lipid and 10% protein by weight. The lipid portion contains various phospholipids as well as neutral lipids such as cholesterol. There are four Surfactant Proteins (SP) that make up the protein portion of LS. They are SP-A, B, C, and D. Previously, various LS lipid and protein components have been studied to understand how they interact with the lipids and with each other (Reviewed by Johansson & Curstedt, 1997; Veldhuizen *et al.*, 1998).

Lipids in LS are approximately 80-90% phospholipids (PL) and some lipids may exist in different structural polymorphic forms. The most abundant PL is phosphatidylcholine (PC) which constitutes approximately 80% of most mammalian surfactant. The major PC that is present in LS is dipalmitoylphosphatidylcholine (DPPC) and it is this PC that gives rise to the surface activity of LS, enabling γ to be greatly reduced. To allow this, the DPPC films become tightly packed in LS to reduce γ to low values (Reviewed by Goerke, 1998; King & Clements, 1972; Nag, 2005; Possmayer *et al.*, 2010). DPPC is also an unusual disaturated (16:0/16:0) acyl chain neutral phospholipid not found in most other mammalian membranous systems. This suggests that it may have a special role in LS. Phosphatidylglycerol (PG) and phosphatidylinositol

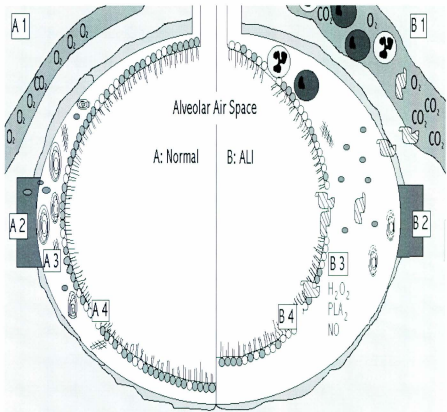


Figure 1: Diagram of a normal alveolus (A2-A4) compared with an alveolus suffering from ALI. During ALI, serum proteins and other material may become inserted in LS (B2-B4) and possibly replace DPPC with less surface active phospholipids or proteins (Bailey & Veldhuizen, 2005) (Reprinted with permission from authors and publisher).

(PI) are two other charged phospholipids present in LS. Other PL include phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM), lysophosphatidylcholine (LPC) and lyso-bis-phosphatidic acid (LBPA) which are also present in minor amounts (1-3% by weight) in most mammalian LS. However, some studies have begun to suggest that mass spectral analysis of dysfunctional surfactant has quite a different composition than surfactant found in normal lungs (Heeley *et al.*, 2000).

Neutral lipids, mainly cholesterol, some triglycerides and free fatty acids are also present in LS. Cholesterol is present in significant amounts, approximately 8-10% by weight (about 20 mol %) of total lipids (King & Clements, 1972; Serrano & Pérez-Gil, 2006). It has been previously suggested that cholesterol aids in increased fluidity which allowed surfactant to cover more surface area and increased the rate of adsorption of DPPC in LS to the air-water interface (Goerke, 1998; Taneva & Keough, 1997). Cholesterol is responsible for fluidity, but excess cholesterol may be problematic because it prevents PL from tightly packing in LS films upon compression. It may also limit the ability of LS to reduce γ to low values (Gunasekara *et al.*, 2005; Leonenko *et al.*, 2007; Lopez-Rodriguez *et al.*, 2011; Veldhuizen *et al.*, 1998), which is the reason why most clinically used surfactants, such as Bovine Lipid Extract Surfactant (BLES) used in this study have the cholesterol component removed.

1.3 Surfactant Film Structures

Although the status of the complex films at the lung air-aqueous interface *in situ* is not clear to date, a group of *in vitro* studies over the last decade provides some significant information about structures formed in such films related to surface activity. In some

earlier studies, it was shown that surfactant lavage from porcine, bovine and other animal lungs, when compressed at an air-water interface, undergo phase segregation of lipids into a gel-like (condensed) and a liquid crystalline (fluid)-expanded phase (Nag *et al.*, 1996-2010; Reviewed by Possmayer *et al.*, 2010). Atomic force and fluorescence microscopy, mass-spectral imaging and grazing angle x-ray diffraction (GAXD), showed that the gel like domains or phase had differential tilt and conformation compared to the fluid phase (Amerin *et al.*, 1997; Harbottle *et al.*, 2003; Keating *et al.*, 2007, 2011; Nag *et al.*, 2002; Worthman *et al.*, 1997; Zasadzinski *et al.*, 2001, 2005; Zuo *et al.*, 2008 a-c). By mass spectral imaging it was shown that the gel-like regions, consisting mostly of DPPC, DPPG and cholesterol, had close association with the gel-lipid domains (Harbottle *et al.*, 2003; Keating *et al.*, 2011; Nag *et al.*, 2008). Other studies showed that cholesterol at normal and excess amounts induced re-arrangement of the gel-lipids and formed multi-layered structures at low γ (Amrein *et al.*, 1997; Gunsekara *et al.*, 2005, 2008, 2010; Leonenko *et al.*, 2007).

Although these studies, conducted *in vitro*, suggest some structural features related to surface activity of natural lung surfactants, the *in situ* situation in the alveolar films is not yet understood. The lipid-protein complexes in the lungs such as lamellar bodies, tubular myelin and the surfactant film are possibly all connected and cannot be simulated *in vitro*, thus the current status of the air-alveolar surfactant is still a mystery. Some studies, however, are suggesting that in the case of experimentally induced lung injury, organized surfactant structures in the lung are dramatically altered (Reviewed by Bailey & Veldhuizen, 2005; Lewis & Veldhuizen, 2003). Monolayers formed *in*

vitro from lavage of such injured lungs also suggest dramatic alterations of domain morphology (Keating *et al.*, 2011, Panda *et al.*, 2004; Ravasio *et al.*, 2010). Some of these studies are currently being extended by addition of various perturbants, as found in serum, to clinically used synthetic and semi-synthetic lung surfactants, to co-relate the structure-(dys)function properties of surfactant from injured lungs (Banerjee, 2002; Gunasekara *et al.*, 2010; Keating *et al.*, 2007; Larsson *et al.*, 2006; Nag *et al.*, 2006, 2010).

1.4 Surfactant Proteins and Lung Disease

Lung surfactant, as previously mentioned, contains four surfactant proteins (SP-). SP-A, B, C and D. SP-A and SP-D are hydrophilic glyco-proteins, while SP-B and SP-C are smaller hydrophobic proteins which interact with the LS lipids. The hydrophobic proteins have been linked to quick adsorption of LS lipids to the air-aqueous interface, increasing the adsorption rates of LS, and promoting a more compressible monolayer film that is highly enriched in DPPC (Reviewed by Possmayer *et al.*, 2001).

SP-A and SP-D are members of a family of hydrophilic proteins called collectins, which are large multi-meric glycoproteins (Reviewed by Johansson & Curstedt, 1997). These proteins have the ability to bind to various microbial ligands and pathogens to help keep the alveolar airspaces in a sterile condition, which increases innate lung immunity (Reviewed by Serrano & Pérez-Gil, 2006). Other studies are consistent with these proposed connections between immunity and collectins. A series of animal studies have been conducted using knockout mice for the SP-A and SP-D genes to decipher their

role in lung disease (Johansson & Curstedt, 1997; LeVine & Whitsett, 2001; Reviewed by Serrano & Pérez-Gil, 2006). Although SP-A has some interactions with SP-B in promoting a TM type surface-active LS complex, surfactant extracted from genetic SP-A knock-out mice showed normal surface activity (Floros & Hoover, 1998; Glasser & Noguee, 2006).

It was noticed, that SP-A and SP-D deficient mice were more susceptible to infection due to the impaired ability of the lungs to remove various microbes by alveolar macrophages. This increased susceptibility to pulmonary infections and increased inflammation (LeVine & Whitsett, 2001; McCormack & Whitsett, 2002; Sano & Kuroki, 2005). These studies may model why these pulmonary infections cause serious disease in humans, for example in ARDS patients there is some deficiency of SP-A and SP-B in their lung lavage (Greene *et al.*, 1999; Reviewed by Hallman *et al.*, 2001, 2002; Whitsett & Weaver, 2002).

SP-B and SP-C are proteins that help enhance the surface activity of the phospholipid films of surfactant. Both proteins are required for lung function and surfactant regulation. SP-B and SP-C purified or in combination, enhance the rate of formation of surface active phospholipid film, *in vitro*, at the air-water interface (Cochrane & Revak, 1991; Hawgood *et al.*, 1998, Weaver & Whitsett, 1991). SP-B is a 17.2 kDa saposin-like protein that is produced from a larger precursor (Whitsett *et al.*, 1995; Whitsett & Weaver, 2002). SP-B was noted to increase adsorption rate of the LS lipids by accelerating the formation of a surface active film of phospholipids (Reviewed by Johansson *et al.*, 1994). Studies by Cochrane and Revak, among others, have

documented that SP-B improves surface activity more than SP-C when added to a PL film (Revak *et al.*, 1986; Yu & Possmayer, 1988). SP-B has been shown to ensure a highly enriched DPPC monolayer and influence the squeeze-out of unsaturated lipids from the surfactant film (Floros & Phelps, 1997; Nag *et al.*, 1999).

Along with SP-B, SP-C also has some role in the formation of the surface active surfactant film. Both SP-C and SP-B have been known to regulate the processes that govern surfactant production and maintain the function of alveolar type II cells (Mulugeta & Beers, 2006). According to Possmayer and colleagues, SP-C appears to have a unique purpose in recycling the surfactant material back into the monolayer film (Possmayer *et al.*, 2001). SP-C functions to improve surfactant surface activity by increasing the rate of adsorption of the lipids to the air-water interface (Johansson *et al.*, 1994; Possmayer *et al.*, 2001; Mulugeta & Beers, 2006). SP-C is the only surfactant protein solely synthesized in the alveolar type II cells and detected only in these alveolar cells of the mature adult lung (Glasser *et al.*, 2001; Mulugeta & Beers, 2006; Wert *et al.*, 1993). Although SP-B knock-out is fatal, SP-C knock-out animals survive with other complications related to lung infections. These studies may suggest that surfactant proteins may function in combinations, yet not clear, especially in their role in disease (Curstedt, 2005; Mulugeta & Beers, 2006; Nogee, 2002).

Levels of surfactant associated proteins in lung lavage can serve as a biomarker in a disease such as ARDS (Greene *et al.*, 1999). In a more recent study it was suggested that serum components may affect the ability of SP-B and SP-C to function properly, thereby inactivating LS (Larsson *et al.*, 2006).

1.5 Inhibition of LS in Disease

In certain diseases, LS can become dysfunctional or inactive. This is evident in various abnormal respiratory conditions. In adults, one condition is known as acute respiratory distress syndromes (ARDS). The term ARDS was first coined by Ashbaugh *et al.* in 1967 and unfortunately not all criteria of such conditions were defined. In 1993, a new definition for ARDS was created by the American-European Consensus Conference (AECC) (Dahlem *et al.*, 2007), this definition described the early onset of the syndrome where respiratory distress in adults was caused by lung collapse and inflammation (Ware & Matthay, 2000). Leaver and Evans (2007) define ARDS as a difficult to treat hypoxemia and accumulation of exogenous substances in the lung which shows up on patient chest cavity X-rays.

ARDS can develop as a result of various underlying conditions, with the highest prevalence in patients with sepsis and septic shock (Leaver & Evans, 2007). ARDS is first suspected when one has difficulty breathing and shows signs of fluid retention (pulmonary oedema) in the lungs (Leaver & Evans, 2007). Inflammation of the alveoli occurs and a fluid rich in proteins leaks into the lung due to increased permeability of the capillaries, which is caused by epithelial damage (Tzouvelekis *et al.*, 2005; Reviewed by Ware & Matthay, 2000). Even in the early stages of ARDS, the alveoli became flooded with plasma proteins which may possibly inactivate LS (Gregory *et al.*, 1991). Lung surfactant of ARDS patients are also shown to contain a decreased percentage of PC, PG and a decreased level of SP-A. Such surfactant has reduced surface activity (Griese, 1999).

In addition to the above, most ARDS patients also show poor lung compliance,

low lung volumes and poor gas exchange (Griese, 1999, Reviewed by Lewis & Veldhuizen, 2003). A study by Gregory *et al.* (1991) compared lung lavage of normal and ARDS patients and found abnormalities in LS composition which indicate that changes in some LS components occurs early in the disease. Their results showed that minimal surface tension (γ) increased in ARDS patients compared to those seen in normal patients. This suggested that the ability of LS to lower γ was compromised (Gregory *et al.*, 1991).

The acute phase of ARDS is characterized by a protein-rich fluid in the air spaces of the lungs, due to a more permeable alveolar-capillary formed from microvascular endothelium and alveolar epithelium damage. The epithelium of the alveoli is composed of 90% (surface area) flat type I cells, which are easily injured and 10% (surface area) cuboidal type II cells (which produce LS) which are less susceptible to injury. Damage to the epithelium, mainly type I cells, can contribute to flooding of the alveoli, impaired removal of edema fluid from the alveolar spaces, and some injury to type II cells which may reduce the surfactant production and turnover (Pugin *et al.*, 1999; Reviewed by Ware & Matthay, 2000). Serum leakage into the airspace from more permeable capillaries is a characteristic of most ARDS patients. Other studies have suggested that serum proteins like albumin, hemoglobin and fibrinogen found in serum, inhibit surfactant function *in vitro*. This became evident when studying the ability of LS to lower γ on the addition of these proteins (Lewis & Veldhuizen, 2003).

Other than serum proteins, cholesterol, lipoproteins, and ions can enter the lungs through plasma and cause surfactant dysfunction in injured lungs. A recent study by

Markart *et al.* (2007) has shown that patients with ARDS have elevated levels of neutral lipids and cholesterol is the main cause of inactivity of the surface films. Other studies have shown a two-fold increase of cholesterol in hyperventilation injured LS of rats, studied as models of ARDS (Keating *et al.*, 2007; Panda *et al.*, 2004). Other studies have shown that during allergen challenge, as in Asthma, molecular species characteristic of those found in plasma lipoproteins are also detected (Heeley *et al.*, 2000; Reviewed by Passmayer *et al.*, 2010).

1.6 Cholesterol Function in LS

Cholesterol has been suggested to play major functional roles in cell membranes (Revised by Ohvo-Rekila *et al.*, 2002). Cholesterol is mainly a rigid, hydrophobic molecule with a polar OH substituent, making it slightly amphipathic in character. The slight amphipathic nature enables anchoring of the molecule to the aqueous or polar interface of the cell membrane. In the cell membrane, cholesterol makes these fluid membranes more rigid. Cholesterol also functions to give fluidity to certain rigid or gel-like lipids (sphingomyelin), to prevent the phospholipids from coagulating and crystallizing. The role of cholesterol in membranes is evident above and below the membrane's gel-liquid phase transition temperature. Cholesterol fluidizes the gel phase and it rigidifies the fluid phase, acting as a membrane buffer (Diemel *et al.*, 2002; Revised by Ohvo-Rekila *et al.*, 2002), leading to formation of a liquid-ordered phase termed as lipid rafts. Cholesterol has been shown to be a major constituent of lipid-rafts in biological membranes, where sphingomyelin, fluid lipids and cholesterol may accumulate for specific protein function (Browne & London, 1998,

2000). Serna *et al.* (2004) have also suggested that the SP-B and SP-C proteins may reside in similar lipid-raft structures in LS bilayers for their function.

Larsson *et al.* (2003) showed that cholesterol actually caused a reduction in the transition temperature of the lipid bilayer of porcine lung surfactant (Larsson *et al.*, 2006). Cholesterol also played some role in LS by helping to improve the ability to spread the DPPC into surface monolayers (Amrein *et al.*, 1997; Panda *et al.*, 2007, 2009). Studies suggest that the addition of cholesterol, in amounts that mimic those found in normal lungs, has no effect on functioning of exogenous surfactants devoid of the lipid. Cholesterol is mainly removed from many clinical surfactants and they seem to function normally *in vitro* and thus the role of this lipid is not clearly known (Keating *et al.*, 2007; Possmayer *et al.*, 2010). Veldhuizen *et al.* (1998) suggested that cholesterol served in the processing and packaging of phospholipids into lamellar bodies and perhaps in their secretion; but may not have had any major role in γ reduction. A recent study by Gunashekara *et al.* (2010) has shown that removal of excess cholesterol from dysfunctional LS by cyclodextrin can restore the normal surface activity of the surfactant.

In order for cholesterol to have any role in the functioning of surfactant, it first must be incorporated in LS during secretion. The lung secretes up to 20% of its total cholesterol in the form of lamellar bodies in surfactant (Hass & Longmore, 1980). The lung is capable of endogenous cholesterol synthesis (Salisbury-Murphy *et al.*, 1966). A study by Hass and Longmore (1980) demonstrated that while the lung is more than capable of producing its own cholesterol, the main mechanism used in the introduction of

the lipid in surfactant is the use of exogenous lipoprotein cholesterol. In a study in 1979, Hass and Longmore reported that only 1% of surfactant cholesterol is of endogenous origin and synthesized in the lung. The rest, they mention, was supplied by serum low (LDL) and high density (HDL) lipoprotein possibly entering through pulmonary arterial blood flow (Davidson *et al.*, 1997; Hass & Longmore, 1979, Hass & Longmore, 1980).

LDL is the major transporter of cholesterol to cells, including the lung, and is required for cellular metabolic and membrane building purposes. A LDL is a spherical, monolayer bound particle with a cholesteryl-ester and triglyceride rich hydrophobic core. The neutral lipids are surrounded by a monolayer of free cholesterol, phospholipids and an Apoprotein B (Apo-B) molecule. The Apo-B in the LDL is recognized by receptors in most cellular membranes. HDL delivers cholesterol to the liver via a process called reverse cholesterol transport and facilitates cholesterol removal from cells and tissues (Tall, 1990). Hass and Longmore, 1980, also reported that receptors for LDL and HDL were present in the lung and suggested that cholesterol metabolism in the lung may be regulated by these lipoproteins (Davidson *et al.*, 1997; Hass & Longmore, 1980).

Competitive binding studies concluded that the lung indeed had receptors for both LDL and HDL. Unlabeled LDL eagerly competed with labeled LDL for binding and addition of HDL resulted in an increase in binding of labeled LDL. Unlabeled HDL was a strong competitor for the labeled HDL. Unlabeled LDL showed competition with the HDL only at high concentrations (Hass & Longmore, 1980). Furthermore, this study showed that deuterated [^3H] cholesterol uptake from both lipoprotein classes into surfactant occurs at least in part, by uptake by the lung, rather

than an exchange since the lipoproteins inhibited endogenous synthesis. This result, coupled by lung surfactant cholesterol inhibition by LDL and HDL, is evidence to show that cholesterol metabolism in the lung was regulated similarly to that in other tissues (Anderson & Dietschy, 1977; Hass & Langmore, 1980).

Even though Hass and Longmore (1980) found that the rate of uptake of the lipoproteins remained similar, the uptake rate for the protein portion of these lipoproteins was different, as LDL apoprotein was found to have a higher uptake rate. This suggested that LDL may be taken up as a whole particle, whereas the cholesterol in HDL may be transferred without definite uptake of the apoprotein component. They further reported that majority of the surfactant cholesterol is derived from lamellar bodies, which are known to be storage sites of surfactant phospholipid components (Hass & Longmore, 1980). A later study by Davidson *et al.* in 1997, provided some evidence that the lung does endogenously produce surfactant cholesterol. There was no major difference of cholesterol effects amounts in any surfactant fraction, even when serum levels were reduced (Davidson *et al.*, 1997).

In 2001, Orgeig and Daniels infused a rat tail vein with [^3H] cholesterol and a large boost in cholesterol specific activity was seen in lamellar bodies (LB) of surfactant within the first 30 minutes. After a two day period, this increase was not seen in the alveolar surfactant, suggesting that the secretion of cholesterol in alveolar space is tightly controlled. Further examination by studying the limiting membrane of the LB showed a high concentration of cholesterol in such membranes compared to the bulk or inner core (Pérez-Gil, 2008). It was concluded that it was unlikely that LB were the major sources

or storage sites of surfactant cholesterol, because the major portion of LB cholesterol was not released into the alveolar surfactant, as the limiting membrane of LB fused with the cell membrane upon exocytosis; therefore, keeping the cholesterol inside the cell plasma membrane (Orgeig & Daniels, 2001). Therefore it is possible that the surfactant cholesterol synthesis occurred within the lung and was stored elsewhere from the phospholipid components. It seems from these studies that there may be other sources of cholesterol in the type II cell (Orgeig & Daniels, 2001). Especially in ARDS or lung injury, this cholesterol may possibly be supplied by leaked serum (Keating *et al.*, 2007; Markart *et al.*, 2007; Panda *et al.*, 2004).

A study by Voyno-Yasenenetskaya *et al.*, in 1993, examined lipoprotein-mediated (receptor mediated) signal transduction and exocytosis of surfactant from the alveolar type II cells of rat lung. They demonstrated that LDL and HDL stimulated signal transduction and surfactant secretion in type II cells. Next, it was determined that alveolar type II cells themselves had receptors for LDL. Specific antisera to the LDL receptor was immunoblotted to freshly isolated type II cells. It was concluded that membranes of the type II cells had LDL receptors, due to a band on a SDS/PAGE that migrated the same distance as the LDL band (Voyno-Yasenenetskaya *et al.*, 1993). This also reaffirmed the previous study by Hass and Longmore, where it was found that HDL and LDL delivered radiolabeled cholesterol first to lamellar bodies from type II cells and then on to secreted surfactant (Davidson, *et al.*, 1997; Hass & Longmore, 1980; Voyno-Yasenenetskaya *et al.*, 1993).

Voyno-Yasenenetskaya *et al.* (1993) also suggested that HDL and LDL

stimulation of surfactant secretion may only occur at a basal level and the level increased due to hormones and other activators, through a signal transduction pathway. These studies showed that the lipoproteins not only supplied cholesterol to surfactant but affected the assembly and secretion of the surfactant from alveolar type II cells.

Previous studies have shown that cholesterol present in the LS films in physiological amounts, has no effect on the ability of LS to reach low values (Gunasekara *et al.*, 2005). The purpose of this amount of cholesterol is unclear, as it seems to have no major effect on surface activity (Leonenko *et al.*, 2007). As seen in diseased and injured lungs, cholesterol present in excess amounts, such as 20 wt %, may affect surfactant drastically, but its removal can restore normal activity (Gunasekara, 2010).

Keating *et al.*, 2007, examined the effect of cholesterol on the biophysical as well as physiological properties of BLES. At normal LS levels similar to those found in a normal lung, it was found that cholesterol had no significant effect on γ reduction, similar to systems with no cholesterol in LS. However, addition of cholesterol above 20% proved to prevent BLES from lowering γ . They also provided evidence that when cholesterol, added in excess to the amount of DPPC, caused formation of a liquid disordered phase, which affected surface activity (Keating *et al.*, 2007). Later studies by the same group has shown the localization as well as structural effects of cholesterol in LS monolayer films (Keating *et al.*, 2011). This study shows that normally cholesterol is localized in gel-domains or in close association with DPPC gel-like regions in surfactant films (Keating *et al.*, 2011). In excess amounts, cholesterol can induce fluid-like

structures trapped in such gel-domains or abolish such gel-domains (Hane *et al.*, 2009, 2010; Keating *et al.*, 2007, 2011; Laing *et al.*, 2009).

These findings supported those of an earlier study by Gunasekara *et al.*, 2005. They also showed the inability of surfactant with a high cholesterol content to lower the minimal surface tension and inability to provide structural stability to the lung. It was determined that cholesterol formed a complex with DPPC and the unbound cholesterol (that was not associated with saturated phospholipids) caused LS dysfunction (Gunasekara *et al.*, 2005). Some of these studies may suggest that serum proteins may not be the only cause of LS dysfunction. Cholesterol either alone or in conjunction with serum proteins may be a major factor in LS inactivation in ARDS.

1.7 Role of Serum Proteins in ARDS

It is known that some serum proteins that leak from the capillaries into the fluid lining the lungs may render LS inactive, but exactly which proteins are involved as well as what mechanism by which inactivation occurs remains unclear. Previous studies on albumin, fibrinogen as well as C-reactive (CRP) protein have been conducted *in vitro* to suggest these proteins somewhat inactivate LS (Greise, 1999; Holm, 1992; Nag *et al.*, 2004; Panda *et al.*, 2004). By using BLES with and without bovine serum albumin (BSA) in low (12.5-250 wt%) and higher amounts (2000-3000 wt%), one study showed that a twenty fold increase of protein to lipid ratio can inhibit surfactant (Vidyasankar, 2004; Hillier, 2005). However, other studies on serum proteins in lung lavages of dysfunctional surfactants have conclusively shown that the soluble protein fractions only increase by two to three fold in injured or ARDS lungs (Günther *et al.*, 2001; Panda *et al.*, 2004).

Thus pathophysiological amounts of serum proteins (20-30 wt%) may not be the main inactivator of LS.

Albumin is a major protein found in serum and is a carrier of fatty acids in blood. Various biophysical techniques such as Langmuir-Blodgett balance, adsorption, and atomic force microscopy (AFM) were used to examine the inactivating effects of BSA (Nag *et al.*, 2006, 2010; Vidyasankar, 2004). It was found that BSA, only in high amounts (200 wt%) prevented γ of LS from reaching low values. The hypothesis to rationalize the high amounts of BSA required was that BSA interfered with lipid packing at the surface of the BLES monolayer film during compression only at high amounts. BSA adsorption to the interface was separate from BLES, as well as BSA may attach somehow to the polar lipid headgroups of the lipids in BLES films (Nag *et al.*, 2010; Vidyasankar, 2004).

Albumin is not the only serum protein known to inhibit LS function. Serum fibrinogen is also proven to inactivate LS (Holm *et al.*, 1999). A study by Devraj, 2005, added evidence that serum proteins do in fact inactivate LS, however at very high amounts. Fibrinogen (Fbg) was mixed with BLES in a range of solutions from 0.1:1 to 5:1, as well as 10:1 Fbg:BLES, wt/wt. The high concentrations, 5:1 and 10:1 were used to show maximum inhibition. By using similar biophysical techniques that were used in the previous study on albumin, the inhibitory effects of fibrinogen were clearly evident. It was seen that fibrinogen had prevented BLES from adsorbing quickly to the air-water interface (Devraj, 2005). Various other studies on albumin, globulin, CRP, and fibrinogen using both extremely high and patho-physiological concentrations of protein have yielded

somewhat complementary results and show minimal inhibition at the patho-physiological levels (Enhörning *et al.*, 2000; Holm, 1992; Liu & Chang, 2002; Otsubo & Takei, 2002). The varying concentrations of protein, various surfactant compositions, and different surface activity measuring techniques make such studies difficult to compare (Holm, 1992).

BLES is a modified clinical surfactant extracted from bovine lungs and is composed of most LS lipids and surfactant proteins, SP-B and SP-C (Yu *et al.*, 1983). By using techniques such as AFM, height differences between the fluid (liquid expanded) and gel (liquid ordered) phases were imaged and measured in BLES films. Fluorescent microscopy, with the use of a fluorescent probe, identified gel domains as dark (probe-free) regions throughout a bright fluid area in such films (Amerin *et al.*, 1997; Nag *et al.*, 2007; Zuo *et al.*, 2008a-c). When these films were compressed, a phase separation (transition) occurred and gel domains, were formed in a “sea” of fluid lipids (Rania & de Kruijff, 2001). The domains, rich in DPPC lipids were possibly tightly packed regions and the fatty acyl chains tilted more perpendicular to air than those in the fluid phase. The gel domains (also called tilt-condensed phase) changed shape and size and disappeared upon further compression, or when excess cholesterol or serum proteins were added to the LS films (Nag *et al.*, 2007).

Devraj (2005) conducted studies examining the structural appearance of BLES and BLES + Fbg films at various γ . The higher the concentration of fibrinogen added, the fewer BLES domains present and at 100% fibrinogen (1:1, BLES:Fbg), domains were non-existent. Through the use of Raman spectrometry studies, it was seen that Fbg

somehow interacted with BLES in the bilayers used to form such films.

Another serum protein used as a biomarker indicative of ARDS is C-reactive protein (CRP). CRP is an inflammatory associated glycol-protein and its levels are known to increase in serum during the inflammatory process and in lung disease (Dong & Wright, 1996). It was mentioned by Nag *et al.*, 2004, that due to CRP ability to bind to PC molecules, this may have become an important factor in surfactant inhibition *in situ* (Nag *et al.*, 2004). CRP can make its way into the lungs by means of serum leakage as well as being produced by lung macrophages.

Drover, 2006, compared BLES and BLES + 10% CRP in several biophysical studies. Results indicated that when CRP was added to BLES, rate of adsorption of LS to the air-aqueous interface was decreased only at high amounts. As indicated by AFM and Raman studies, CRP agglutinated the gel domains and caused an increase in the gel phase domains (Drover, 2006). Findings from Chang *et al.*, 2002, indicated that perturbation of the monolayer or bilayer surface lipid-packing or a gel-fluid coexistence was necessary for CRP binding. The studies suggest there are possibly different mechanisms by which an individual protein may interact (i.e CRP-binding to DPPC) with individual components of LS. In a recent study it was also suggested that serum proteins may cause inhibition of LS by interacting directly with SP-B or SP-C or both (Larsson *et al.*, 2006). However, a recent study has shown that cholesterol can modify the effect of SP-C and its orientation in surfactant films (Gómez-Gil *et al.*, 2009a, 2009b).

1.8 LS and Interaction with Whole Fetal Calf Serum

Previous studies (Reviewed by Griese, 1999; Günther *et al.*, 2001; Holm *et al.*,

1999), including some of the above, have established that individual serum proteins had somewhat detrimental effect on surfactant function only at very high amounts. However, other non-protein factors in serum may affect LS at pathophysiological levels. Fetal calf serum (FCS) is very similar to that of human serum in composition. Not only does human serum contain soluble proteins, like albumin, fibrinogen, CRP and globulins, it also contains various ions, amino acids, sugars, (such as fructose and glucose) as well as serum lipids. These lipids may be in the form of low and high density lipoprotein (LDL and HDL) containing triglycerides, fatty acids, cholesterol and cholesterol esters (Friedlander, 2005).

In a previous study of fetal calf serum with BLES [Hillier, 2005 (Honors Thesis)] we examined the structure and function of BLES with FCS compared to albumin, by using a capillary surfactometer (CS) and a Langmuir balance, respectively. It was observed that a small patho-physiological amount of FCS was needed to render BLES completely inactive compared to 200 fold amount of the soluble protein albumin (Hillier, 2005). Different amounts of FCS and albumin [0.1-2:1 BLES, dry (wt/wt)] on BLES dispersions were studied. As increased amounts of albumin from 10 to 2000 wt% were added to BLES, a greater inhibitory effect took place. With increasing protein from 10 to 50 wt% and at the highest amount, maximal inhibition was seen. The same was seen for FCS with concentrations ranging from 1% to 500 wt% of FCS, but this time maximal inhibition was seen at very low concentration of only 5 wt%. Further studies were conducted at concentrations ranging from 0.1 to 5 wt% FCS. Results indicated that as little as 2 wt% FCS was required to show the same inhibition of BLES as seen with

500 wt% albumin (Hillier, 2005, Nag *et al.*, 2007).

This study provided compelling evidence that whole serum (FCS) was a two hundred times more potent inhibitor of BLES than albumin. From monolayer studies it was seen that serum dramatically perturbed lipid packing of the BLES and prevented a low γ from being reached. Another preliminary study in our laboratory showed that the complete serum protein fraction (devoid of serum lipids) failed to inhibit LS at low amounts (Verge, 2010). As most previous studies have shown that serum protein alone, or its protein fraction, does not alter LS surface activity in a significant manner below high (10-20:1, serum protein:LS) concentrations, this suggests that other components of serum may be responsible for LS inactivation.

Panda *et al.* (2004) published a study reporting that in hyperventilated injured rat lungs (ARDS model) the cholesterol content of dysfunctional surfactants increased two fold compared to normal lungs with a concomitant threefold increase in soluble proteins. Since most studies from our laboratory (Devraj, 2005; Drover, 2006; Parsons, 2005; Vidysankar, 2004) and others (Holm *et al.*, 1992; Warriner *et al.*, 2002) have found that only high levels ((10-20:1) of serum protein:LS) can cause LS inactivation. In this study, serum lipids or lipid fraction were mainly used as the target of our investigation.

To support the aforementioned serum study, Parsons (2005), studied the structural properties of BLES monolayers with varying FCS concentrations (1:0.01-1:1, BLES: serum, dry wt/wt) as a preliminary investigation of how lung surfactant structures are altered. Serum inhibition of BLES was studied using a Langmuir surface balance, to see if surfactant's intrinsic ability to lower surface tension was compromised,

while use of both fluorescence and atomic force microscopy examined the film ultra-structure at different surface tensions. The preliminary microscopy studies demonstrated that whole serum, at lower concentrations, disrupted larger gel-domain formation and caused smaller and more numerous domains to form (Nag *et al.*, 2007; Parsons, 2005), however these structural changes were very different from those induced by albumin (Nag *et al.*, 2010). At higher concentrations, serum had the ability to form its own film and prevented lipids from occupying any space in the film and inhibited surfactant lipid adsorption (Nag *et al.*, 2007; Parsons, 2005).

1.9 Purpose of Present Study

Since serum as a whole did indeed inactivate LS at very low concentrations compared to serum proteins alone, there are possibly other components of serum that cause this inhibitory effect. However, exactly which of the non-protein components are involved is not clear to date. Examining other serum lipid components and observing their effects on surface activity and structure of LS would be a significant contribution towards understanding the physiological relationship between serum and LS. The present study investigated the functional and structural properties of BLES bilayer dispersions and monolayer films on the addition of serum lipids (cholesterol and LDL). BLES (pure) and BLES: whole serum, cholesterol and LDL, at various ratios (1:0.1 and 1:0.2, BLES:serum or cholesterol or LDL, dry wt/wt) were examined by a Langmuir surface balance, through compressing adsorbed films formed from bilayer dispersions of the mixtures. Also, detailed Iatroscan, Electrospray and MALDI-TOF mass spectroscopy were performed on BLES, serum lipid and LDL to understand the exact lipid composition

of these materials.

Isotherms of surface tension (γ) vs. area were plotted and minimum γ and compressibility of such films were measured to understand change of functional aspects of surfactant with serum and serum lipids. Atomic force microscopy images of the deposited films were obtained in high resolution, to comprehend the structural changes in such films at different surface tension induced by serum components. To examine the effect of these serum lipids on the bilayer dispersions used for the film formation, Raman spectroscopy was used to obtain order parameters and to understand the effect of such serum materials on bilayer packing and chain conformations, at different temperatures. The Raman studies allowed us to compare the condensed-fluid phase transitions in BLES-serum lipid films with these thermally induced gel-liquid crystalline transition in bilayers.

Chapter 2: Materials and Methods

2.1 Materials

Samples were prepared with Bovine Lipid Extract Surfactant (BLES[®]) dispersion, with and without 10 and 20 wt% of Fetal Calf Serum (FCS), cholesterol, and LDL. The clinically used BLES (27mg/ml) was a generous gift from Dr. Dave Bjarnson of BLES[®] Biochemicals Inc. (London, Ontario, Canada) and used without further purification. FCS (7mg/ml), LDL (5mg/ml), and cholesterol (solid-crystalline form) were all purchased from Sigma-Aldrich Inc. (St. Louis, Montana, USA) and used as received. Mica sheets used in making AFM/Langmuir Blodgett film deposits were kindly donated by Dr. Erika Merschrod (Department of Chemistry, Memorial University of Newfoundland). These sheets have previously been used as atomically flat surfaces when the mica was freshly cleaved. HPLC grade solvents (99%), chloroform and methanol, were purchased from Fisher Scientific (Ottawa, Ontario, Canada).

Most surface activity and structural experiments were conducted using doubly glass distilled water (ddH₂O), with the second distillation being performed using dilute potassium permanganate (KMnO₄). KMnO₄ was used in the second distilling process to remove any organic, surface active, components and volatile hydrocarbons in air by means of oxidation, to prevent surface contamination for film formation (Nag *et al.*, 1992, 1998). Most samples (except AFM) were studied in 0.15 M NaCl-Trizma[®].HCl buffer, pH 7, made using ddH₂O to prepare such buffers. Glassware used was perchloric acid washed, rinsed with ddH₂O, and dried for 2 hours at 180 °C to remove any organic (oily-lipid containing) surface active impurities (Keough *et al.*, 1988).

The NaCl-Trizma[®].HCl buffer used in this study was made with the addition of 150mM (~9 g) NaCl to 5 mM (~0.08g) Trizma hydrochloride and dissolved in 1 liter of double distilled water and adjusted to pH 7 by titrating with addition of 0.1M NaOH solutions. All buffer adjustment solutions as well as acids and alkali were also prepared using ddH₂O. These protocols have previously been used in our laboratory for the last two decades for optimal surface activity measurements impurity removal (Kcough *et al.*, 1988; Nag, 1996; Nag *et al.*, 2007, 2008).

Samples were prepared by incubating desired amounts (10 and 20 wt%) of FCS and LDL with appropriate amounts of BLES dispersion and mildly vortexed to ensure adequate mixing. Small aliquots were diluted appropriately with buffer to be used for all monolayer and bilayer model studies. This was done to ensure that the similar stock of samples was used in all experiments and to avoid heterogeneity of sample concentration and composition, which otherwise would make the comparison difficult.

2.2 Lipid Extraction of Samples

For AFM, Raman and mass spectrometry studies of BLES and BLES+ 10 and 20% cholesterol, BLES was extracted in hydrophobic solvents from aqueous dispersions by the use of the Bligh and Dyer method (1959). Briefly, in this method, 0.8 volume of BLES dispersion (27 mgs/ml), two volumes of methanol, and one volume of chloroform were combined into a graduated tube and mechanically mixed by shaking. Following this, one volume of chloroform along with one volume of double distilled water was added and again vortexed to ensure that the solutions were well mixed. This mixture was centrifuged for 2-3 minutes at 1000 rpm to separate the organic and aqueous

layers. The organic phase at the bottom layer was extracted into a glass vial with a glass pipette as to not disturb the layers and to prevent re-mixing. The remaining aqueous layer was remixed using the same volume of chloroform as organic material and the organic solvent layer was further extracted. Again the organic layer was collected in the glass vial. This extraction was repeated with 2:1 chloroform:methanol and all organic extracts were pooled into a single vial. The organic solution was placed under nitrogen gas to dry, then left overnight in a dessicator to remove any trace solvents.

Cholesterol, on the other hand, needed to be added to BLES differently.

Cholesterol is a non-polar crystalline solid and did not mix easily with an aqueous solution. Even though cholesterol contains a polar hydroxyl group, the larger, ringed portion of the molecule is non-polar, giving rise to its hydrophobic characteristic. Once the BLES extracts were dried and weighed on a microbalance, 10 and 20% of cholesterol by weight, in 3:1 chloroform:methanol, was added to the dried extract, re-dissolved in the same solvents, mixed, and dried under nitrogen and dessicated. For use in this study, the BLES:cholesterol mixture was re-suspended in a saline buffer, by vortexing above 30 °C, to provide for multi-lamellar vesicles as observed using electron microscopy by methods discussed elsewhere (Nag *et al.*, 1998, 2004, 2007).

To help identify the lipid components of both FCS and LDL, the lipid portions of each sample were extracted by using the Bligh and Dyer method (Bligh and Dyer, 1959). The organic layer of each were dried under nitrogen and the residue was then used for analysis by Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS). Each sample was mixed with 0.5mol/L 2,5-

dihydroxybenzoic acid (DHB) matrix, which worked best for lipid materials (Nag *et al.*, 2006). The difference in sample preparation was attributed to the fact that both serum and LDL samples were purchased already in aqueous solutions, therefore they were able to mix with the supplied BLES bilayer dispersion samples.

2.3 Methods

For all experiments conducted in this thesis, such as adsorption, monolayer, AFM and Raman, BLES and BLES +10 and 20% serum or cholesterol or LDL mixtures were used. These amounts were pathophysiologically relevant to those seen in disease, as well as seen in the normal lung surfactant lavages, as in the case of 10% cholesterol (Panda *et al.*, 2004). Adsorption, monolayer balance and AFM studies were used to investigate the structure and function of monolayer films, whereas the use of Raman spectroscopy was used to examine the bilayer phase of these mixtures. All experiments were conducted at 24±1°C, with the exception of Raman spectroscopy, where temperature variation studies (10-40°C) were conducted. The adsorption, monolayer film and AFM studies were performed at a monitored ambient room temperature.

2.3.1 Analysis of Lipid Components (ESI- MS & MALDI-TOF)

Previous studies using Matrix Assisted Laser Desorption-Ionization time of flight (MALDI-TOF) mass spectrometry (MS) and electrospray-ionization MS (ESI-MS) have shown specific componential distribution of surfactant lipids and proteins in porcine, bovine and rat LS (Harbottle *et al.*, 2003; Nag *et al.*, 2007; Panda *et al.*, 2004). Serum materials involved in surfactant dysfunction can also be characterized. MALDI allows for

direct detection and characterization of individual lipid species of extracted surfactant of normal and diseased lungs. In this study we used an Applied Biosystems Voyager System 1027 (Voyager-DETM-CA) spectrometer to analyze the lipid components of BLES, LDL, and serum lipid extracts to specifically measure the estimates of cholesterol and cholesterol esters in these samples, as well as some studies were followed up using Iatroscan. In MALDI-MS, 100 μ l of chloroform:methanol was dissolved and samples were injected in the spectrometer and the spectrum obtained between 100-1000 m/z (Nag *et al.*, 2004). The mass spectral profiles were obtained as intensity as a function of mass/charge (m/z), where the charge (z) is normally 1. MALDI-TOF allowed us to use the samples directly without addition of other ions, which is a normal problem encountered in ESI-MS where some other ions are detected (Harbottle *et al.*, 2003; Nag *et al.*, 2007).

We compared the MALDI-TOF data with Iatroscan as methods of detecting lipids using chromatography. The Iatroscan method combined thin-layer chromatography and flame ionization detection (Schrijver & Vermeulen, 1991). With this method, a lipid mixture (serum), was separated using silicic acid coated quartz rods and then quantified using flame ionization detection (Kramer *et al.*, 1985) to measure the lipid classes present.

2.3.2 Adsorption

Adsorption experiments were conducted using a small cylindrical Teflon cup with a volume of 5 ml and a surface area of 6.28 cm^2 (Nag *et al.*, 1996). BLES and BLES + 10 and 20% serum or cholesterol or LDL dispersions were injected by a Hamilton syringe

through a small rubber valve into the cup underneath a clean buffer surface. Samples were constantly stirred using a magnetic stir bar. Adsorption of sample to surface was determined by the drop in γ which was monitored as a function of time in seconds, by using a Wilhelmy-platinum dipping plate. This method was followed using a protocol described in detail by Nag *et al.*, 1996, 1998.

2.3.3 Monolayer Balance Studies

In order to study the γ lowering abilities of serum, cholesterol and LDL, a Langmuir-Wilhelmy balance (Applied Imaging, London, England) was used to produce a surface tension-area monolayer isotherm for adsorbed films. This balance had an area of 500 cm², which was large enough to allow compression of films to lower γ values very close to 1 mN/m. A motorized leak-proof rectangular Teflon tape barrier compressed and expanded the monolayer films while a platinum Wilhelmy plate hung from a force transducer detected the change of γ (Nag, 1997; Nag *et al.*, 2007; Taneva & Keough, 1994).

Prior to beginning each experiment, the trough and Teflon tape barrier were thoroughly cleaned with chloroform:methanol (3:1) solution and then with ddH₂O. The solutions were each suctioned off and the trough was dried. The trough was filled with ddH₂O and BLES and BLES + 10 and 20 wt% samples of serum or LDL or cholesterol (system) dispersion were adsorbed to form films with an initial γ drop close to 60 mN/m by adsorption. Compression and expansion of monolayer films was conducted at a rate of 2 mm²/sec. Compression allowed the lipid monolayer to undergo a fluid to gel phase transition. Details of the Langmuir-Blodgett method using this trough were previously

published (Nag *et al.*, 2007; Taneva & Keough, 1994).

This balance was also used to deposit films using Langmuir-Blodgett methods at various γ on mica disks for AFM studies (Nag *et al.*, 1999). The monolayer films were compressed to γ values of 52 mN/m, 42 mN/m, and 32 mN/m respectively. Mica discs, used for depositing films that were previously inserted in water, were vertically lifted out of the ddH₂O subphase at a rate of 1 mm²/sec to provide Langmuir-Blodgett films (Nag *et al.*, 2003). We used a standard surface tension-percent film area protocol for plotting our data instead of the standard surface pressure-area per molecule isotherms. This was done as a result of technical difficulties in calculating the exact area per molecule of “adsorbed” films as well as with comparing the γ data with some previous studies using captive bubble methods, where area/ molecule data was redundant and could not be calculated (Gunaskara *et al.*, 2005). The details of the techniques and physics of the Langmuir Balance and trough have been discussed previously (Nag, 1991; Nag, 1996).

2.3.4 Atomic Force Microscopy (AFM)

Deposits consisted of BLES and BLES + 10 and 20 wt % serum or LDL or cholesterol were used for structural studies using AFM. A Teflon dipping head, which held three round freshly cleaved mica disks, was lowered into the water subphase before the sample film was made. Compression (2 mm²/sec) from ~60mN/m to stepwise decrease of three desired surface tensions (52, 42 and 32 mN/m, respectively) was conducted and the compression stopped during the deposition process. Upon reaching the desired γ , the dipping head was slowly retracted at a rate of 1 mm²/sec.

Nag *et al.* (2004b) provided further details on surfactant film studies using the AFM of Langmuir-Blodgett film imaging.

By using AFM, films were imaged with an Nanoscope IIIa atomic force microscope (AFM) (Digital Instruments, CA). In brief, AFM used a small tip, attached to a cantilever and the sample base laterally moved across the surface of the deposited films in contact mode. This vertical movement of the tip during scanning, determined height differences as well as surface features present in the sample films (Zasadzinski *et al.*, 2001). This was done via the deflection of a laser off the end of the cantilever. The deflection changed based on attraction or repulsion of the tip by the surface, as well as due to horizontal inhomogeneity on the surface. The monolayer film surface showed these inhomogeneities at nano-scale (0.5-2 nm) due to formation of condensed and fluid domains, as well as protein aggregates. Sample topography images were seen when the laser deflection was plotted versus tip position (Nag *et al.*, 2007, 2008; Zasadzinski *et al.*, 2001).

Both samples were imaged within two hours from the films being deposited to prevent dehydration (Nag *et al.*, 2004). These deposits were imaged in air with a Nanoscope IIIa scanning probe (in Contact mode) microscope (Digital Instruments, CA) with a silicon nitride tip (Nag *et al.*, 2007). The field sizes imaged were anywhere from 5 μm x 5 μm to 20 μm x 20 μm without zooming into the low resolution image. All images were processed with Nanoscope IIIa software to give 2D, 3D and friction mode images as discussed elsewhere (Nag *et al.*, 2003). We also used another MFP (Molecular Force Probe)-3D AFM for some follow up studies and comparison with our AFM data.

For the BLES + 10 and 20% LDL samples, images were taken using a MFP-3D AFM (Santa Barbara, CA), in Contact mode, and using a built in flexured scanner. Again field sizes as above ranged from 5 μm x 5 μm to 20 μm x 20 μm . These images were processed using IGOR Pro software (WaveMetrics, Portland, Oregon, USA), with the help of Dr. Erika Merschrod, MUN Chemistry, to produce 2D, 3D and sectional images, similar to those obtained using Nanoscope IIIa software. The two different AFM's worked differently. The Nanoscope system allowed for the sample base to move during scanning and the MFP-3D tip moved laterally with a stationary base.

To easily compare domain sizes and heights, each field size, Z-scale and deposited surface tensions of all samples were kept reasonably close, to the best of our ability. All monolayer films were prepared and deposits made from three ($n=3$) independent sets of studies. At least 3-4 random spots were scanned for each sample and the best representative images were displayed. All AFM deposits were made on films formed on a doubly distilled water surface (no buffer). This protocol was used since the salts present in buffer may dry during imaging and cause crystallites of the salts to disrupt sensitive film architecture (Nag *et al.*, 2004).

2.3.5 Raman Spectroscopy

The technique of Raman spectroscopy relies on the light scattering induced by polarization changes in the electron density in vibrating molecules. In Raman spectroscopy, an energy change occurs, either as a gain or a loss of photons, which is characteristic of certain bond vibrations from different parts of the molecules, or, in other words, functional groups. It is characterized by frequency shifts of incident

photons due to specific vibration of bonds of the compounds at a frequency range of 10^{12} seconds. Raman spectroscopy can suggest specific bond vibrations in a single phospholipid system or an average of signals from specific bonds (C-H, C-C, PO₄) in a complex system such as BLES.

In Raman spectroscopy, normally a visible laser of 520 nm is focused on the sample, and the transmitted photons are detected for frequency shifts due to the bond vibrational energy of the molecules in the sample. In our Raman Spectral-microscope (RMS) [LABRAM-K, Horiba Jobin Yvon, Edison, NJ, USA], a green solid-state laser beam is focused through a microscope confocal objective on the surface of the sample held in a heating-cooling small Teflon container with glass bottom (200µl in volume). The scattered photons from the sample are collected through the same objective and detected by a cooled-CCD (Raman-filtered) detector. The software (LabSpec) of the RMS and filters in the microscope provide for the shift in the frequency of the scattered photons and the data plotted as intensity as a function of wavenumber (Raman-shift) in cm^{-1} .

Temperature studies were used to examine various vibrational modes of BLES and BLES + 10 and 20% serum or cholesterol or LDL samples at temperatures ranging from 10–40 °C, taken at increments of 5 °C. Any changes in shape and position of the bands were indicative of the phase changes and perturbants on BLES bilayers. The temperature range was chosen to study BLES since previous studies have shown BLES to undergo a broad gel to liquid crystalline phase transition between 10–40 °C with a midpoint around 27 °C using DSC and ²NMR (Nag *et al.*, 2006).

In another study, it was also shown that these gel domains of BLES can be visually observed using fluorescence microscopy of giant unilamellar vesicle bilayer close to the midpoint of the transition (Nag *et al.*, 2002). The bands that were of most interest were those in the 2800-3000 cm^{-1} range. These were mainly the C-H symmetric and asymmetric stretching (vibrational) bands mainly from the fatty acyl chains hydrocarbons in the C-C skeletal backbone of the chains of phospholipids. CH_2 symmetric and asymmetric stretching modes are the strongest detected in the spectra of lipids and are at 2850 cm^{-1} ($\nu_s \text{CH}_2$) and 2890 cm^{-1} ($\nu_{as} \text{CH}_2$) respectively (see **Figure 2**).

Changes in these bands corresponded to changes in the *trans/gauche* conformation ratios in the acyl chain. This can also be seen, though not as clearly, for changes due to terminal CH_3 symmetric (2930 cm^{-1}) and asymmetric (2960 cm^{-1}) stretches (Spiker and Levin, 1975). Other bands that can be examined are the phosphate stretches found in the 700-900 cm^{-1} range from phospholipid head groups. These, however, are very weak in nature (Wong & Mantsch, 1985), due to a single PO_4 bond as well as the water of hydration associated with the head groups. A cartoon of these phospholipid vibrational modes are shown in **Figure 2**.

The Raman Spectral microscope (RMS) used was a LABRAM confocal microscope (Horiba Jobin Yvon, Edison, NJ, USA) with a grating of 1800 groves/mm. A Leica microscope with a long-working distance objective is attached, which has a magnification factor of 50X, and a Peltier CCD detector. A 532nm green laser (diode pumped solid state laser) line was excited to yield spectra. Each spectra had a accumulation time of anywhere between 6-20 seconds to find the best spectra and were

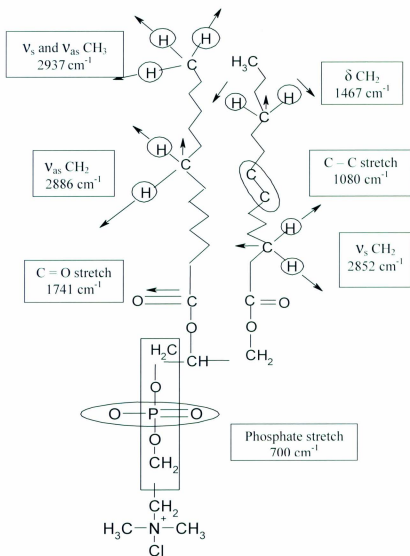


Figure 2: A cartoon of a typical phospholipid molecule which shows the various group vibration (stretches) in the acyl chain and head group regions that can be observed using Raman spectroscopy (modified from Deveraj, 2005).

acquired using the D₀ filter. The short time (1 minute per temperature) was chosen to prevent sample evaporation and drying. A similar method has been previously described in detail (Nag *et al.*, 2003). Samples were examined by using a small glass cuvette sample chamber placed on the stage of the microscope which was controlled by a water heating/cooling system (5-50 °C).

Chapter 3: Results

3.1 Chemical Composition of Serum and Surfactant

To identify lipid components of whole FCS, both MALDI-TOF and Iatroscan were used. BLES extract was also used in MALDI-TOF mass spectrometry. **Figure 3** shows the spectra for (a) BLES and (b) serum lipids. Evident from the spectra (a), BLES showed an intense peak at 735 m/z (molecular weight of DPPC + H⁺). This is the parent ion peak for DPPC, confirming that BLES contains mostly DPPC. The other peaks in the 700+ m/z range are other phospholipid classes especially the fluid 16:0/18:1 PC at 761 m/z. The smaller peaks between 400-500 m/z were possibly fragments of lipids and fatty acid conjugates. The next step was to investigate which lipid classes were in serum. The serum (solvent extract) spectra revealed a variety of lipids but showed the highest intensity peak as cholesterol ion at 369 m/z as appropriated from other studies. Cholesterol is not detectable as a whole molecule, but detectable only upon the elimination of water or the OH group (Reviewed by Fuchs & Schiller, 2009; Schiller *et al.*, 2001). Small amounts of sphingomyelin and other fluid phospholipids were also detected and could possibly be remnants of lipoprotein or other membranous systems present in serum (i.e. 16:0/18:1-PC is a major component of lipoprotein shells and red blood cells) (**Figure 3(b)**). The spectra for LDL suggested the presence of high amounts of cholesterol esters as expected (496 Da), however, showed a significant amount of free cholesterol (370 Da). We are not clear if this free cholesterol is from breakdown of cholesterol esters during mass spectral ionization or from the membranous outer monolayer of LDL, since some phospholipids from the outer membranes (704-788 Da)

were also detected (**Figure 3(c)**). This is confirmed from the low amounts of free cholesterol (6%) detected by Iatroscan (**Table 1**). Free fatty acids and triacylglycerols (TAG) were also detected using this method.

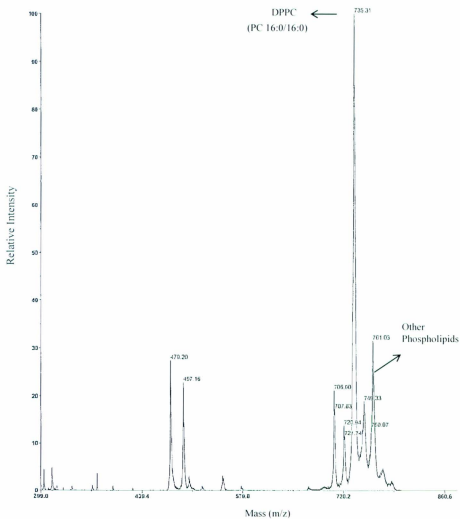
The data from the Iatroscan (IS) of serum was shown in **Table 1** where the serum lipids were extracted from the FCS and the lipid composition percentage from each lipid class. This data gave a complete lipid analysis and was recorded as % lipid composition. The highest amounts of lipids were sterol esters (37%-cholesterol ester) and phospholipids (21%).

3.2 Adsorption Isotherms

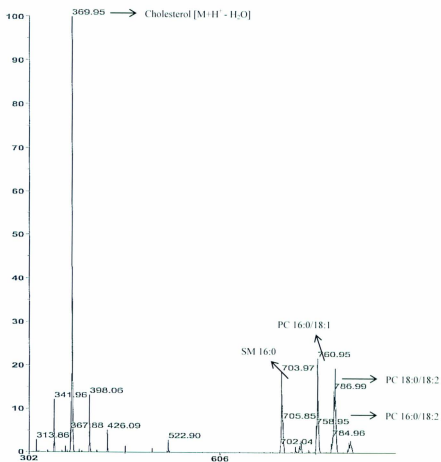
Adsorption experiments were conducted with dispersions of BLES (13.5 mg/ml). Serum, LDL and/or cholesterol mixtures with BLES were injected under a clean air-water interface. Measurements of lipid adsorption over time (seconds) were recorded by monitoring γ continuously for 300 seconds. Two different amounts (10 & 20% wt.) of serum, cholesterol and LDL were mixed with BLES dispersions with the total volume being kept constant by dilution with buffer. **Figure 4** shows the adsorption curves for BLES and BLES additives. Each adsorption curve represented the average data obtained from three replicate experiments, with the standard deviation represented by error bars.

Pure BLES adsorbed at the water interface (γ ~20 mN/m) within 100 seconds. Addition of serum and LDL to BLES reduced the rate and reached γ of ~50-55 mN/m even after 300 seconds. Unlike the other samples, cholesterol addition to BLES lowered the γ to ~30mN/m, which was not significantly different than that of pure BLES.

(a)



(b)



Mass (m/z)

(c)

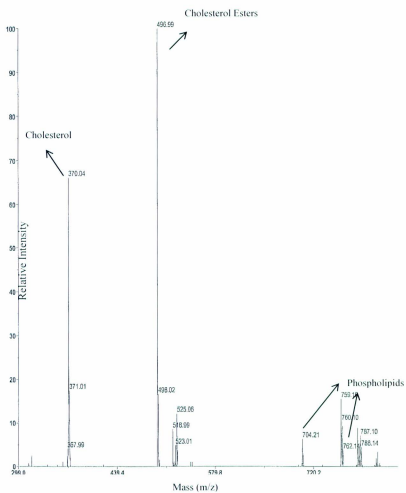


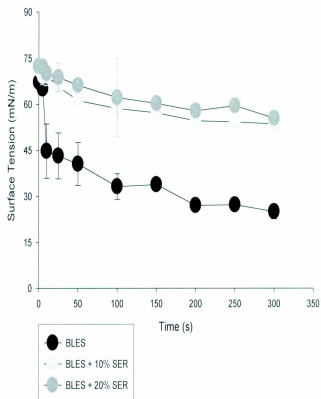
Figure 3: MALDI-TOF mass spectra of (a) BLES and (b) serum lipids (c)

LDL. For the BLES sample, DPPC was the most abundant. As for the serum lipid extract, cholesterol was the most abundant. Several other phospholipid classes are detected in the 700-800 m/z range, and could be from the membranous outer layer of LDL. (see text).

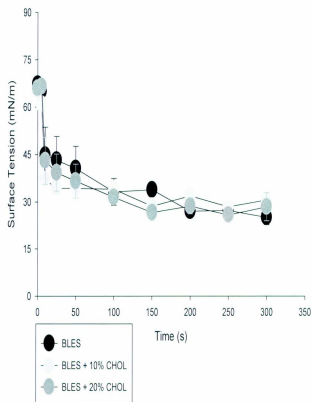
Table 1: Lipid Classes and Their Percent Lipid Composition Detected in Serum Lipid Analyzed by Iatrosan

Lipid Classes	Lipid Composition (%)
Hydrocarbons	5.9
Steryl Esters/Wax	37.9
Ketones	2.9
Triacylglycerols	6.5
Free Fatty Acids	11.5
Alcohols	4.9
Sterols	6.1
Acetone Mobile Polar Lipids	3.3
Phospholipids	21.0

(a)



(b)



(c)

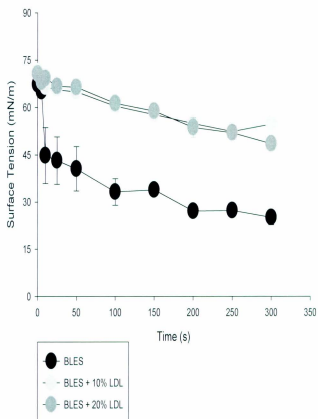


Figure 4: Adsorption isotherms (γ vs. time) of BLES dispersions with 10 and 20% (a) serum, (b) cholesterol, and (c) LDL at $24 \pm 1^\circ\text{C}$. Each plot is an average of 3 independent experimental sets. Standard deviation is shown by the error bars of $n=3$ experiments. BLES adsorbed to a γ of $\sim 20\text{mN/m}$ after 300 seconds, whereas with serum and LDL, they only drop to a γ of $\sim 55\text{mN/m}$, even after 300 seconds. Cholesterol in BLES (10 or 20 wt%) showed γ drops close to those of pure BLES ($\sim 30\text{ mN/m}$), suggesting cholesterol does not significantly affect the adsorption rates.

Also serum and LDL did not allow BLES to rapidly adsorb (to equilibrium γ) to the air-water interface. It should be noted that physiological amounts of cholesterol (10 or 20%) had no significant effects on adsorption. In **Table 2** the tabulated values of minimum γ reached after 300 seconds showed a slight increase of 3 mN/m compared to γ after 150 seconds for samples containing cholesterol. However, these values are within the limits of experimental error.

3.3 Monolayer (Langmuir) Balance Studies

Multiple compression and expansion cycle isotherms of BLES and BLES with serum, LDL and cholesterol of the adsorbed films of the same mixtures in **Figure 4** were conducted in the Langmuir surface balance. The same concentration of sample lipid (100 $\mu\text{g/ml}$) per group was adsorbed in each of the three trials. The monolayers were formed by initial adsorption of the dispersions to an air-aqueous interface. Compression-expansion cycles were carried out at a speed of 2 mm^2/sec after initial adsorption of the films to -62 mN/m (**Figure 5**). Each experiment was conducted in triplicate, the data averaged, with the standard deviation represented by the error bars. **Figure 5** compares compression-expansion isotherms of (a) BLES in buffer with (b) 10% serum, (c) 20% serum, (d) 10% cholesterol, (e) 20% cholesterol, (f) 10% LDL, and (g) 20% LDL. BLES films reduced the γ to a minimum of near -1 mN/m values from an initial γ of 60 mN/m for all cycles. Upon addition of serum to BLES, there was a drastic increase in the minimum γ reached (30 mN/m) at maintained compression. This increase in γ was similar to that also observed upon the addition of LDL (**Figure 5(f)**).

Table 2: Tabulated Average Adsorption Time Values for BLES With and Without Additives.

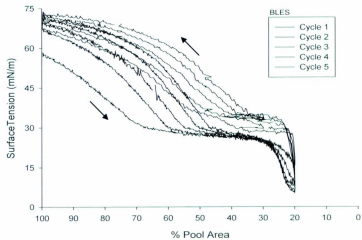
Adsorbed Sample Films	Surface Tension at 1, 150 and 300 sec of Adsorption to Water Interface		
	γ_1	γ_{150}	γ_{300}
BLES	71 ± 0.5 mN/m	35 ± 0.5 mN/m	25 ± 1 mN/m
BLES+10% Serum	73 ± 1 mN/m	58 ± 1 mN/m	53 ± 1 mN/m
BLES+20% Serum	74 ± 0.5 mN/m	61 ± 0.5 mN/m	59 ± 0.5 mN/m
BLES+10% Cholesterol	70 ± 2 mN/m	21 ± 0.5 mN/m	31 ± 1.5 mN/m
BLES+20% Cholesterol	65 ± 0.5 mN/m	26 ± 0.75 mN/m	27 ± 1.25 mN/m
BLES+10% LDL	73 ± 0.5 mN/m	59 ± 0.5 mN/m	56 ± 0.75 mN/m
BLES+20% LDL	72 ± 0.5 mN/m	59 ± 0.5 mN/m	50 ± 0.5 mN/m

Results are from n=3 experiments. The \pm sign indicates standard deviation of the mean. A slight increase (± 3 mN/m) was observed in the samples containing cholesterol after 300 seconds.

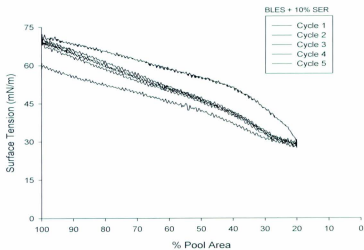
It was evident that serum, LDL and excess cholesterol interfered with the ability of BLES films to lower γ upon compression. BLES films were shown to reduce γ to a minimum of near ~ 1 mN/m values. Upon addition of serum to BLES (**Figure 5(b) and 5(c)**), there was a drastic increase in the minimum γ (γ_{\min}) to 30 mN/m compared to the low values for pure BLES. Changes in γ_{\min} were also observed upon the addition of similar amounts of LDL (**Figure 5(f) and 5(g)**). Addition of cholesterol in physiological amounts (10%) gave γ_{\min} values comparable to BLES ($\gamma_{\min} \sim 1$ mN/m) films, see (**Figure 5(d) and 5(e)**) above. When cholesterol was added in excess (20%) the γ_{\min} reached was higher (15 mN/m) which showed that excess cholesterol affected BLES ability to reach a low γ . However, this effect was not as significant as that of serum or LDL. It was evident from this that serum, LDL and excess cholesterol impaired the ability of BLES films to lower γ .

With the inhibitory effect on BLES observed with excess cholesterol, whole serum (FCS) was also tested in the monolayer balance. The isotherms of BLES + 10% serum and BLES + 20% serum were shown in **Figures 5(b) and 5(c)**. The inhibitory effect was clearly evident in both. There was no plateau area change in either BLES + 10% or BLES + 20% serum, suggesting that this material did not undergo ‘squeeze out’ and therefore could not be removed easily even after multiple cycling. Histograms of the minimum and maximum γ that were attained during five cycles of compression and expansion of films are shown in **Figure 6**. Each experiment value shown in **Figure 6(b)** represented three replicate experiments, where initial values of γ close to ~ 60 mN/m increased to ~ 70 mN/m after five cycles, mainly in the last cycle.

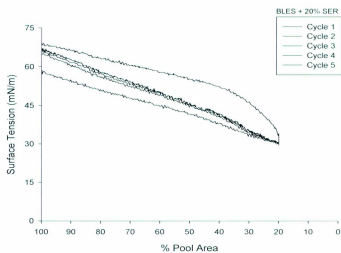
(a)



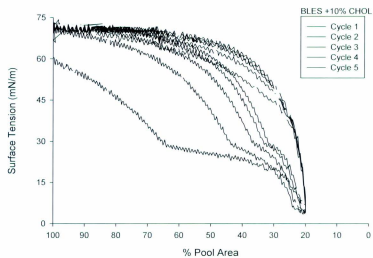
(b)



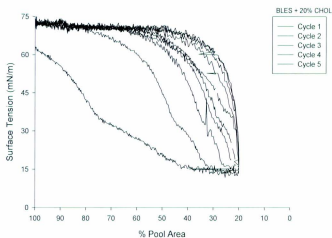
(c)



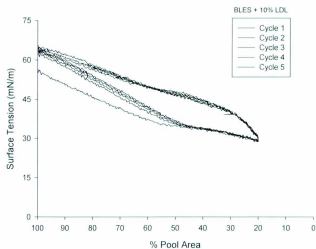
(d)



(c)



(f)



(g)

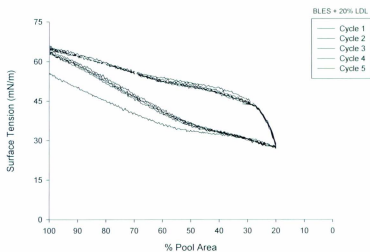
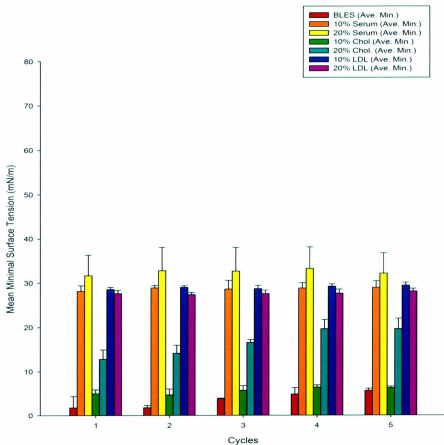


Figure 5: Surface tension-area isotherms of 5 cycles of dynamic compression-expansion at a rate of 2mm²/sec for (a) pure BLES and with (b) 10% serum, (c) 20% serum, (d) 10% cholesterol, (e) 20% cholesterol, (f) 10% LDL and (g) 20% LDL films. Each experiment was conducted in triplicate and the best representative graph from a single experiment is shown for clarity. All isotherms were plotted as percentage of film area change versus surface tension (γ). The downward arrow shows the direction of compression and the upward of expansion. Averaged data of γ from all experiments is shown by the bar graphs in **Figure 6**. The small fluctuations in the γ data were mainly caused due to the vibrations of the compressing Teflon barrier as well as to the hypersensitive pressure transducer of the Wilhelmy apparatus.

As various materials were added to BLES, and films compressed, they reached a minimum γ that increased past that of BLES with $\gamma < 5$ mN/m. The data suggested that BLES films with low values of γ were fully functional and surface active. Experimental data shown in **Figure 6(a)**, obtained upon addition of 10 and 20% serum to BLES, increased the minimum γ to ~ 28 and ~ 30 mN/m respectively, clearly demonstrating that serum significantly prevented the surface activity of BLES films to reach low γ . **Figure 6(a)** further showed that contrary to data obtained on serum containing films, the addition of 10% cholesterol showed a minimum γ close to that of BLES alone.

The fact that natural amount of cholesterol present in surfactant of the lung does not significantly alter BLES surface activity was consistent with our experimental data. Increasing the amount of cholesterol to 20%, increased γ_{\min} to ~ 15 mN/m, however, the effect was not as significant as that of serum or LDL. LDL concentrations, both 10 and 20%, gave rise to high γ_{\min} values close to that for serum around 28 mN/m. Even though 20% LDL had the highest γ_{\min} , LDL was a potent inhibitor of BLES surface activity. Compressibility of BLES and BLES + serum or cholesterol or LDL films was calculated by obtaining C_{15} values as discussed elsewhere (Nag *et al.*, 2004; Panda *et al.*, 2004). The term C_{15} gives compressibility of the sample films which helps in understanding the decrease of film area required to decrease a fixed amount of γ . Briefly, the C_{15} values suggest the total film area (compressibility) required to drop γ of the films by 15 mN/m during film compression from an initial value of 45 mN/m (**Figure 7 (top)**). This calculation can be applied to dysfunctional as well as normal LS films as previously discussed in detail by others (Nag *et al.*, 2004; Panda *et al.*, 2004).

(a)



(b)

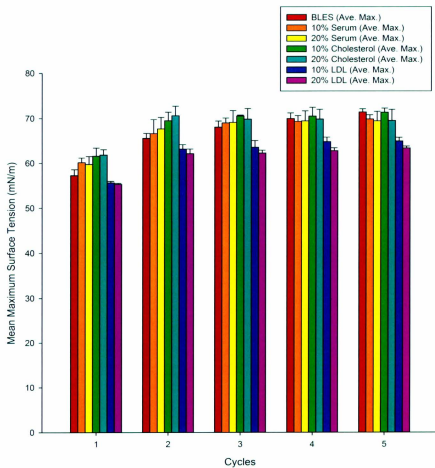


Figure 6: Effects of serum, cholesterol, and LDL on (a) minimum and (b) maximum surface tension achieved by BLES films for 5 cycles of compression-expansion. Three sets of independent experiments were performed and the graphs are indicative of the mean of each sample ($n=3$). The standard deviation is represented by error bars.

Previous studies have shown that for dysfunctional films of LS, larger area compressions are required to drop γ of equivalent amounts compared to significantly smaller areas required for native surfactant or BLES alone (Panda *et al.*, 2004). Large area compression indicated that probably materials which inhibit LS surface activity cannot be easily removed from the surface film either by 'squeeze-out' or by subphase vesicle formation. Serum as well as other components (soluble proteins and cholesterol) are difficult to remove from dysfunctional surfactant by repeated cycling and thus such films cannot achieve low γ , even after multiple cycling (Nag *et al.*, 2004).

As shown in **Figure 7 (top)** the C_{15} values were calculated from a γ change from 45 to 30 mN/m only since serum and LDL films could not be compressed below 30mN/m even at maximal compression. This was the main reason we chose the high γ range (45-30 mN/m) for all films, instead of lower γ regime (>30 mN/m) found in pure BLES films. As shown in the bar graph (**Figure 7 (bottom)**), in order to lower BLES films γ by 15 mN/m, the monolayer had to be compressed only by 15% area (highly compressible). The greater the percent (%) area change, for the equivalent drop of γ , the more incompressible the films were. The lowest compressibility (or highest incompressibility) was seen in the samples of serum and LDL where the films required 45 and 30% compression respectively. When cholesterol was added to BLES in the films, these films were still harder to compress than pure BLES. It was easily seen that to drop to the same level of γ , BLES + LDL and BLES + serum films needed to be compressed two to

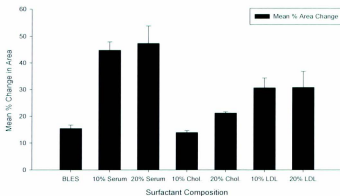
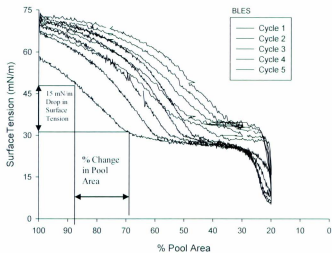


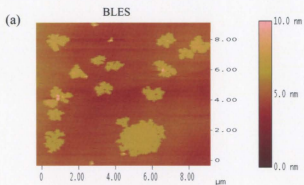
Figure 7: Effects of serum, cholesterol and LDL on compressibility of BLES films based on mean % pool area compression required for a surface tension drop of 15 mN/m (C_{15} values). Serum and LDL containing BLES films had to be compressed twofold more in area than the ones of pure BLES. Error bars represent standard deviation of ($n=3$) 3 independent experiments. The large plateau areas of the isotherms indicate the “squeeze-out” region of the films.

threefold to achieve equivalent γ drops.

3.4 Atomic Force Microscopy (AFM) Structural Studies

The samples used in the monolayer compression-expansion and adsorption studies were deposited on freshly cleaned mica sheets (Langmuir-Blodgett) and examined using contact mode AFM. The images shown in **Figure 8** were representative images of BLES and BLES with 10 and 20% serum or cholesterol or LDL films compressed to a γ of 52 mN/m prior to being deposited on mica. It was evident in the images that upon compression, some of the films underwent a phase transition from fluid (liquid expanded) to gel (liquid condensed) phase (Nag *et al.*, 2007). The gel domains were formed upon compression of films from an initial surface tension of ~ 60 mN/m compressed to 52 mN/m for BLES. In **Figure 8(a)**, the BLES domains were brighter (or higher than surrounding phase), compact and more circular in shape. This is caused by the tilt of the lipid molecules becoming more perpendicular to the plane of the air-water interface in the gel phase. The darker region, which was the fluid phase, was lower in height by about 0.8-1.2 nm.

Based on representative section analysis of corresponding images (as in **Figure 10**), the height of the BLES gel domains was shown to be ~ 1.2 nm higher than the fluid phase. Comparing **Figure 8(a-g)**, it was noticeable that even though all images were taken at a γ of 52 mN/m, there was a large difference in surface heterogeneity possibly due to more complex composition of the films. BLES gel domain formation was clearly disrupted in films with the additives. In **Figure 8(b)** and **(c)** addition of serum tended to form areas with holes as well as some areas with spiked (sharp height differences)



$\gamma = 52 \text{ mN/m}$ for all deposits.

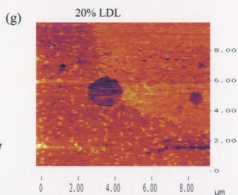
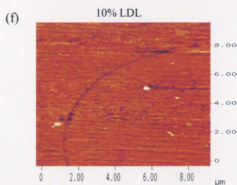
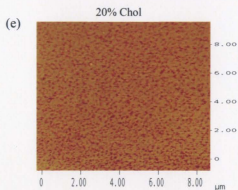
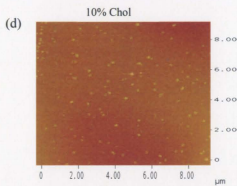
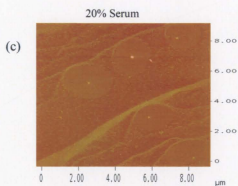
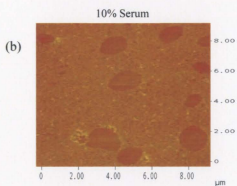


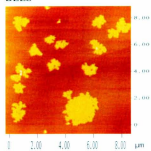
Figure 8: Representative AFM contact mode images of Langmuir-Blodgett films deposited at an equivalent γ of 52 mN/m for (a) BLES and with (b) 10% serum, (c) 20% serum, (d) 10% cholesterol, (e) 20% cholesterol, (f) 10% LDL, and (g) 20% LDL. Films were deposited at a rate of 1 mm²/sec by vertical upstroke. Image field sizes shown were taken at 10 μ m x 10 μ m (x-y plane) and height differences (z plane) were shown by the 10.0 nm bar, indicated in (a). The higher domains were about 0.8-1.2 nm above the lower fluid regions. The bright regions represent gel domains and other micro-domains \sim 1 mN/m higher than the surrounding phase and were also seen in films containing cholesterol (d) and LDL (g).

domains in the center. **Figure 8(d)** showed that with the addition of cholesterol (10%) very small (micro) domains were present, which were lost with the addition of higher amounts (20%) of cholesterol **Figure 8(e)**.

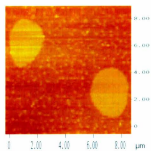
As later shown in **Figure 9**, 10% cholesterol at further compression (γ of 42 mN/m), larger domains were formed with spiked centers. **Figure 8(f)** showed that there were no gel or other domains (hole-like) formed at all. These films showed a solid homogenous appearance with various sized micro-domains dispersed randomly. Looking at **Figure 8(f) and (g)**, LDL addition prevented BLES domain formation and upon addition of 20% LDL, other domains were formed with some fluid-like holey areas. It was easily seen that with the addition of serum or its constituent, there was a dramatic perturbation of BLES gel domain formation and this possibly affected these films ability to achieve low γ .

As evident in **Figure 9(a)**, as the BLES monolayer was further compressed to lower γ , the BLES domains became more circular and larger in size. In addition, small micro-domains appeared in the fluid phase. Upon addition of 10% serum in **Figure 9(b)**, holes were formed with regions of either very high height (1-5 nm) or spiked structures found in the middle of the holes. Upon further compression to a γ of 32 mN/m, large round holes with scattered spike domains were again evident. However, there was very little contrast between the holes and surrounding areas in the AFM images. Addition of 20% serum also shows the formation of deeper but smaller holes, which get larger upon further compression to 32 mN/m. These “holes” may be domains of a lower height than surrounding phase.

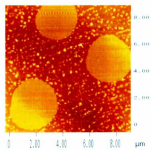
(a) BLES



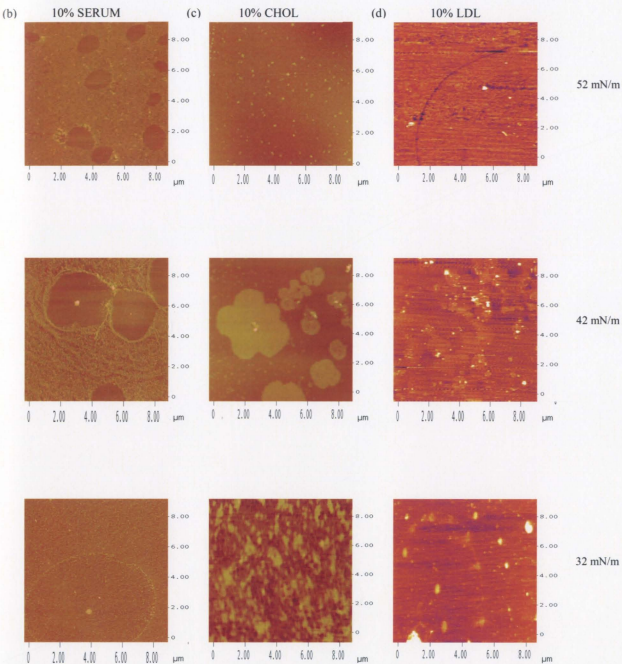
52 mN/m



42 mN/m



32 mN/m



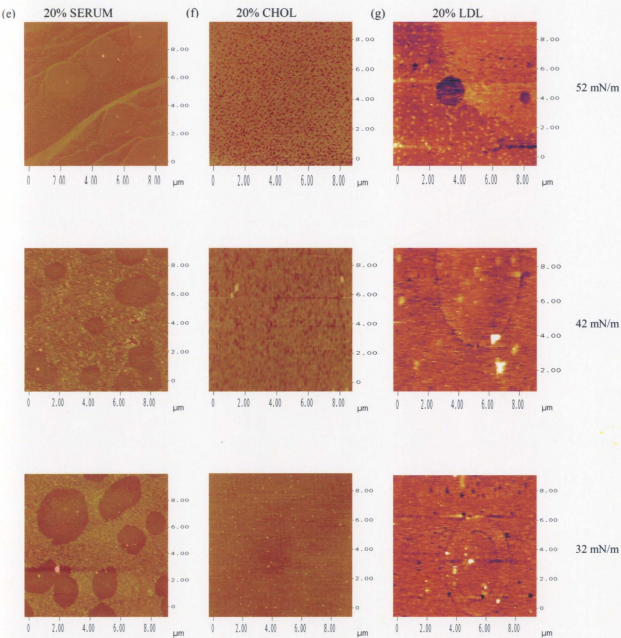
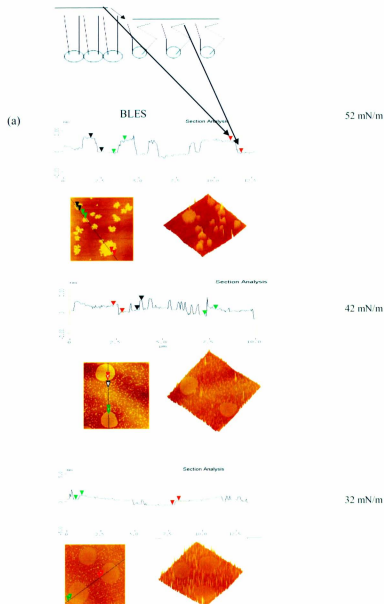


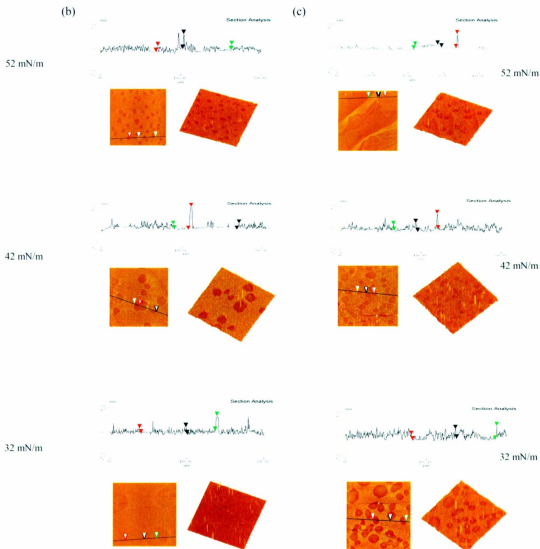
Figure 9: Representative AFM images of films of (a) BLES and BLES with (b) 10% serum, (c) 10% cholesterol, (d) 10% LDL, (e) 20% serum, (f) 20% cholesterol and (g) 20% LDL. All images are shown in 2D and are representative of 3 or 4 different 10 x 10 μm areas taken for each deposit. Deposits are taken at γ 52, 42 and 32 mN/m respectively shown in vertical panels on the right. Upon addition of materials to BLES films, the gel domain (bright circular areas) formation was altered. In (e) and (g) the domains formed were lower in height compared to the surrounding phase, suggesting that these circular domains may be fluid in nature. The vertical height bar (not shown) is the same (10 nm) as **Figure 8**.

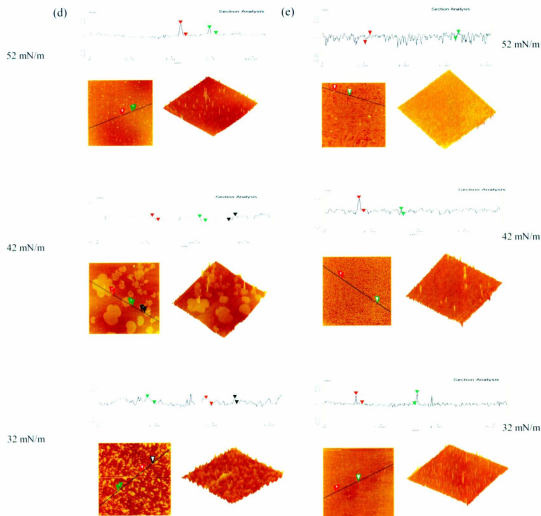
These domains disappeared with further compression of the films and with addition of 20% cholesterol. LDL addition, however, abolished BLES gel domain formation in films and when further compressed, circular and spiky domains, as well as holes were present. This was evident in BLES with 10% and 20% LDL films. These results showed that somehow serum, cholesterol and LDL perturbed the organization and surface morphology of the phospholipid domains in the films and therefore possibly prevented effective packing of lipids in such domains.

Upon further observation of **Figure 10** which shows 3D and 2D sections of each film at γ 52, 42, and 32 mN/m respectively, we see that the 3D images clearly indicate height differences in these films between domains and holes. For BLES (**Figure 10(a)**), gel domains as high as 1.6 nm were formed in a sea of surrounding fluid phase. When serum was added to BLES, hole like structures were seen with some spiky structures inside the domains (**Figure 10(b)** and **(c)**). The holes were ~ 2 nm in depth and the spiky domains were seen to be at a height range of 4-7 nm. When cholesterol was added in smaller amounts as in (**Figure 10(d)**), further micro-domains were formed and became larger upon compression. Spiky centers were formed which disappeared upon further compression and the film became more homogenous. These micro-domains were close to about 1 nm high compared to the intermediate phase between them.

Cholesterol added in excess (20%), prevented domain formation to occur, and the homogenous films were again observed. There was not much difference in height of the structures in the film, except for a few areas with sharp perturbances. Addition of LDL to BLES caused the formation of hole-like regions (1-2 nm deep) as well as some compact







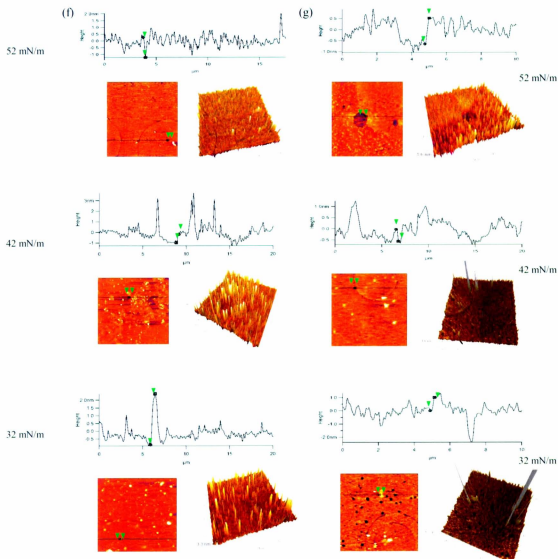


Figure 10: Height differences (section analysis) between the gel and fluid phases of the AFM image of BLES + additive films at γ 52, 42, and 32 mN/m for (a) pure BLES and BLES with (b) 10% serum, (c) 20% serum, (d) 10% cholesterol, (e) 20% cholesterol, (f) 10% LDL and (g) 20% LDL. Both three dimensional (3D) and sectional analysis done on the images as well as height differences are suggested in the plots. Since the LDL film images were obtained using a different AFM (see page 32 for details) the (X-Y) axis did not match with the other images. Also far higher (1-5 nm) height differences (surface roughness) or heterogeneity in the z axis were observed for such films compared to the others.

gel-like domains. In low amounts, LDL prevented BLES gel domain formation but may also have formed domains of its own (~1-3 nm in height). Examination of **Figure 10** revealed that the height difference between the two phases (gel and fluid) gave a clear picture of the phase change.

Previous studies on native extracted surfactant films from rat, porcine and bovine sources have shown these films to contain compact gel domain structures, and such films can be compressed to reach very low γ (1-4 mN/m) (Nag *et al.*, 1998, 2002; Panda *et al.*, 2004). However, in some studies on dysfunctional surfactants, extracted from hyperventilation of injured lungs, it is suggested that large hole-like structures and an absence of gel domains, prevented such films to reach γ below 20-30 mN/m (Panda *et al.*, 2004). It was not known from these studies what the large hole-like structures were, however they have been speculated to either be made of serum-soluble proteins or cholesterol, as these components were also found in excess in the dysfunctional surfactant (Panda *et al.*, 2004).

3.5 Raman Spectroscopy Bilayer Studies

In previous studies in our and other laboratories, Fourier Transform Infra-Red (FTIR) spectroscopy was applied to surfactant systems (Dluhy *et al.*, 1989; Mendelsohn *et al.*, 1989; Nag *et al.*, 2008; Vidyasankar *et al.*, 2004), however the broad water band which appears between 2000-3000 cm^{-1} obscured the sharp CH_2 and CH_3 bands. After the spectral subtraction of water, the interpretation of some of the FTIR data was not tenable due to the complex pattern of bands. Although some of the vibrations did not scatter light well, the CH_2 , CH_3 , and C-C skeletal bands could be clearly defined.

Temperature dependent shifts in some of the bands allowed for a relative measurement of the phase transition of BLES and BLES with additives (Devraj, 2005; Nag *et al.*, 2008). This phase change monitoring allowed for correlating some of the structural features observed in films (due to the 2D lateral phase transitions induced by compression) with those that occurred in the bulk phase since the films are formed from such dispersions. The correlations may suggest structure-function correlations of secreted surfactant below the lung air-water interface with the surface film.

In the spectra of the lipid class (**Figure 11**), the strongest peaks that were mostly evident in BLES were the bands from 2800-3000 cm^{-1} . These were the CH_2 symmetric stretching and asymmetric stretching modes at 2850 cm^{-1} and 2890 cm^{-1} respectively (see **Figure 2**). These bands were sensitive to the *trans-gauche* conformational changes in the acyl chains of lipids. Another Raman band of similar interest was that of the CH_3 symmetric stretch (2930 cm^{-1}) and asymmetric stretch (2960 cm^{-1}). These give information from the interior core of the bilayers. The Raman spectra of BLES and BLES with serum, cholesterol or LDL in the 2800-3000 cm^{-1} region at different temperatures are shown in **Figure 11**. These Raman peaks, for specific phospholipid and their bond vibration designations, have been determined previously by others (Devraj, 2005; Dluhy & Mendelsohn, 1988; Spiker & Levin, 1975; Wong & Mantsch, 1985). As shown in **Figure 11**, the symmetric CH_2 stretch ($\nu_s \text{CH}_2$), at 2850 cm^{-1} , the asymmetric stretch ($\nu_{as} \text{CH}_2$), at 2890 cm^{-1} and the symmetric CH_3 stretch ($\nu_s \text{CH}_3$), at 2930 cm^{-1} are shown for BLES and BLES with 10 % serum or 10% cholesterol or 10% LDL systems. For BLES,



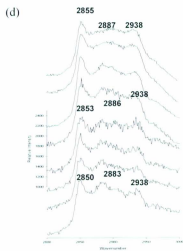
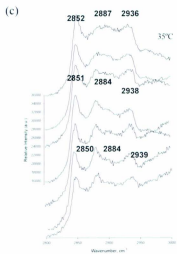
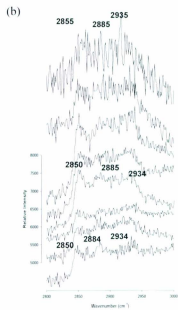
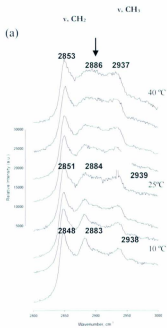


Figure 11: Raman spectra of the 2800-3000 cm^{-1} range for (a) BLES, (b) BLES + 10% serum, (c) BLES + 10% cholesterol, and (d) BLES + 10% LDL. Each graph is a representative of three different experimental trials. Similar trends were shown for BLES and BLES + 20% serum, 20% cholesterol, and 20% LDL samples. Temperature ranged from 10-40 °C and was chosen since BLES shows a broad thermotropic (gel to liquid crystalline) phase transition between the range as well as a midpoint of transition at 27 °C as determined previously by Raman and differential scanning calorimetry (Nag *et al.*, 2006, 2008). Note the shift of the 2848 cm^{-1} band at 10 °C to 2855 cm^{-1} at 40 °C. The shift is characteristic of the BLES system, melting from a gel to liquid crystalline phase. The spectra in (b) had a large noise to signal ratio, which was similar to the ones observed for serum. This could be due to protein auto-fluorescence from the serum soluble proteins.

in **Figure 11(a)**, the ν_{as} showed a trend for a broadening of the ν_{as} CH₂ band with an increase in temperature from 25-40 °C. For temperatures lower than 25 °C, a narrowing of the band was observed.

Another trend noticed was a shift in wavenumber of the ν_{as} CH₂ peak with an increase in temperature. This was similarly observed in **Figure 11(b)-(d)**. Also evident was the intensity of the ν_{as} CH₂ peak compared to the ν_s CH₂ peak. By observing the high temperatures it was evident that the intensity of ν_{as} CH₂ is less than ν_s CH₂ and it increased as the temperature decreased. The ν_s CH₃ peak at 25 °C had less intensity value than that of the ν_{as} CH₂ peak; and as temperature increased so did the ν_s CH₃ intensity.

These trends were consistent with a change in conformation of the lipids as a function of temperature, where an increased fluidity of the hydrocarbon chain or gauche conformation was observed at higher temperatures. At lower temperatures (10-25 °C) however, the hydrocarbon chains were more “gel-like” or rigid with trans conformation. The effects of serum, cholesterol, and LDL on BLES however are not seen quite clearly from **Figure 11**, possibly due to significant signal/noise ratio ascribed to serum protein auto-fluorescence or from apoproteins present in LDL. We did not observe any clear cut peaks from pure serum and LDL due to their high protein content.

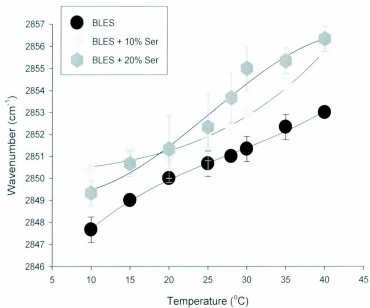
The temperature induced changes in the wavenumber of the ν_s CH₂ band of BLES with serum or cholesterol or LDL are shown with those of BLES in **Figure 12(a-c)**. By plotting the shift of the wavenumber of the ν_s CH₂ peak versus temperature, changes in ordering of the lipids are seen in **Figure 12**. The wavenumber of the ν_s CH₂ (2850 cm⁻¹) was plotted versus temperature for the average of three independent

experiments, the error bars represent standard deviation. These plots can suggest the order parameters of the phospholipid chains as discussed in details elsewhere (Dluhy et al., 1989; Nag, 2008). An increase in wavenumber indicates more fluidity or higher number of *gauche* bonds, whereas lower shift indicates more *trans* bonds or more ordering or rigidity.

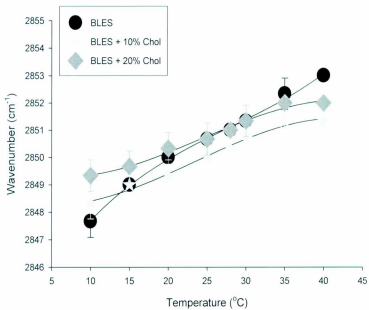
For the BLES curve, a more sigmoidal shaped plot was observed due to a broad phase transition observed between 10–40 °C, the midpoint of transition being about 27 °C, which was measured previously by Differential Scanning Calorimetry (DSC) (Nag et al., 2006, 2008). **Figure 12, (a) and (c)** showed the fluidizing effects of serum and LDL, respectively, on BLES. This was due to an increase in shift to higher wavenumbers, for all points on the curve throughout the temperature range. With the addition of cholesterol, however, the shifts of wavenumbers were not as evident when compared to BLES alone. There was a slight shift to higher wavenumbers for the 20% cholesterol system, however, the effects were not significant, suggesting cholesterol may have rigidified as well as fluidized the system depending on the temperature

However, the systems containing LDL (**Figure 11(c)**) and serum (**Figure 11(a)**), clearly showed a shift of all wavenumbers to higher values suggesting a fluidizing effect of the BLES system with these additives. Therefore, it could be concluded that both serum and LDL fluidized the bilayer, which in turn prevented formation of gel domains in films formed from such bilayers and may have affected LS function. Cholesterol seemed to increase fluidity of the gel phase at lower temperatures and to rigidify the fluid phase at the higher temperatures. The Raman studies directly showed that serum and LDL

(a)



(b)



(c)

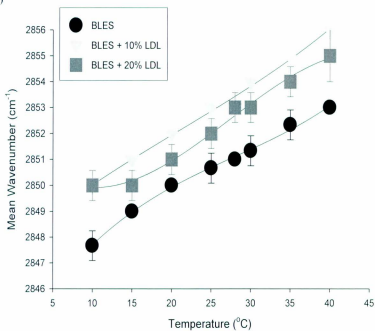


Figure 12: Vibrational Raman wavenumber shift for BLES compared to BLES with (a) serum, (b) cholesterol and (c) LDL. The temperature range was from 10–40°C for all graphs. Each graph is the mean of wavenumber shifts from three independent sources and the error bars represent the standard deviation. Higher shift in wavenumber indicates more fluidity or increasing number of *gauche* bonds, whereas lower shift indicates more *trans* bonds or more rigidity of the hydrocarbon chains (Dluhy et al., 1989; Vincent *et al.*, 1996). Both serum and LDL increased fluidity of the BLES films, while cholesterol had slight fluidizing (10%) and rigidifying or ordering (20%) effects.

affected surfactant packing in bilayers and this information can be related to the structure-function properties in monolayers.

Chapter 4: Discussion

Most studies published over the last three decades on surfactant inhibition by serum proteins have been conducted using mainly surface tension techniques. These studies suggest that inhibition by soluble serum proteins may be major factors in surfactant inactivation in acute respiratory distress syndrome (ARDS). Serum proteins including albumin, fibrinogen as well as C-reactive protein (CRP), among others, have been shown to inhibit surfactant function by inhibiting adsorption and ability of such surfactant films to reach low γ using multiple surface analytical techniques (Devraj, 2005; Drover, 2006; Holm *et al.*, 1992, 1999; Nag *et al.*, 2007; Panda *et al.*, 2004; Vidyasankar, 2004).

However, some studies have shown that serum protein inhibition is concentration dependent as in the case of albumin, and that such inhibition can be overcome with an increase in surfactant to serum protein ratios (Banerjee, 2002; Holm, 1992). Since multiple surface techniques, as well as a variety of protein concentrations were used in these studies, it is difficult to compare such studies with those performed using whole serum, as well as surfactant from ARDS or injured lungs.

To understand the molecular mechanisms involved in ARDS, a number of studies over the last decade have focused on serum lipid components, mainly cholesterol (Diemel *et al.*, 2002; Finot *et al.*, 2010; Gómez-Gil *et al.*, 2009a, 2009b; Gunsekara *et al.*, 2005, 2008, 2010; Hane *et al.*, 2010; Keating *et al.*, 2007, 2011; Laing *et al.*, 2009; Larsson *et al.*, 2003; Lopez-Rodriguez *et al.*, 2011; Nag, 2008; Panda *et al.*, 2007; Serna *et al.*, 2004; Vockeroth *et al.*, 2010). Some of these studies have suggested interesting structure-

function changes in surfactant films with normal and excess cholesterol, and can easily be compared to our studies with serum and LDL, and also be extended to surfactant bilayer or bulk phases.

Cholesterol and Lung Surfactant

Previous studies have shown that cholesterol is an integral component of LS, found to be 7-15% in various mammalian systems (Reviewed by Daniels *et al.*, 1998). However, the physiological role of cholesterol is not clear, since the source of the material as well as its delivery route in LS has not been firmly established. Previous studies also indicate that radiolabeled cholesterol and LDL are taken up by type II pneumocytes, but its insertion in the secreted surfactant pool occurs by a complex process.

Some authors have shown that LDL cholesterol is mainly enriched in the outer lamellar body limiting membrane, and this membrane does not reach the secreted surfactant pool during exocytosis (Orgeig and Daniels, 2001). Jones *et al.*, 1993, has suggested cholesterol is segregated into two types of domains in porcine surfactant, containing SP-B/C (Serna *et al.*, 2004). It is not clearly known what the major role of the lipid is in LS function, let alone in dysfunction. However, in the studies by Gunasekara *et al.* (2008) it has clearly been shown that a normal amount of cholesterol does not affect the surface activity of LS.

Other studies have shown that cholesterol actually allows for formation of specific monolayer to bilayer squeeze-out phases (Gunasekara *et al.*, 2005), as well as allows for function of surfactant proteins SP-B and SP-C by forming lipid-raft like

structures (Bernadino *et al.*, 2009; Serna *et al.*, 2004). Some of our studies agree with the structure-function changes induced by normal cholesterol, however, if excess cholesterol mainly deactivates BLES, whether this cholesterol comes from serum or LDL is not clear to date. There are some differences in interaction of LDL and whole serum with BLES compared to pure cholesterol and our studies tried to compare these interactions.

We are also uncertain about the source of extra cholesterol from LDL and serum. Our mass spectral data (**Figure 3**) does not provide a clear cut idea about excess cholesterol in L.S. The bulk of cholesterol in LDL and probably in serum is supposedly to be found as cholesterol esters. The MALDI-TOF data shows that the cholesterol is mainly detected in free form in the serum lipid extract as well as LDL. The main peak in the mass spectra (**Figure 3**) at 369 m/z is possibly either the fragmented form of cholesterol ester or free cholesterol from some other source. Previous mass spectral study by Schiller *et al.*, 2001, of LDL also suggested that the 369 m/z peak is a deoxygenated cholesterol. [Molecular Weight (M.W.) of cholesterol – OH= 386 – O(16)= 370 or cholesterol + H⁺], and is the major peak. The cholesterol ester peak at 671 m/z was very small as well as contained a Na⁺ ion and this was difficult to detect in MALDI-TOF of LDL (Schiller *et al.*, 2001). However, our Iatroscan data, along with the MALDI-TOF spectra for serum LDL, clearly suggests that the bulk of serum lipids (**Table 1**) contain mainly steryl esters (cholesterol esters) and phospholipids. A more recent study has shown that cholesterol esters are more potent inhibitors of BLES than free cholesterol (Panda *et al.*, 2007, 2009).

Serum Lipids and Proteins

As our earlier studies suggest, serum was discovered to be a more (~200 times more) potent inhibitor than some of its constituent proteins. Concentrations of serum as little as 2 wt% added to BLES dispersions could inactivate BLES in ways that would take ten fold higher soluble protein concentrations or 500-1000 wt% (Hillier, 2005). This gave some credence to the idea that there may be some other factors in serum that caused such potent inhibition, perhaps a lipid-like, non-protein material. A previous study by Panda *et al.*, 2004, focused on the alterations of lung surfactant composition as well as surface activity that occurred during lung injury. Not only were protein levels increased three-fold but levels of cholesterol were also increased about two-fold in such surfactant. Other studies confirmed this and showed that an elevated level of cholesterol from 10 to 20 wt% completely inactivates surfactant such as BLES (Gunasekara, 2005; Leonenko *et al.*, 2007).

In our study, a group of correlated techniques namely adsorption, monolayer balance, AFM and Raman spectroscopy, helped us to produce information at a molecular level of the BLES (surfactant) and BLES with additive (dysfunctional surfactant) systems. It is through the use of these techniques that we were able to examine possible ways that surfactant is effected by serum as well as its specific lipid components in bilayer and monolayer films. Some studies have suggested that LDL may be responsible for excess cholesterol in the lung (Anderson & Deitschy, 1977; Davidson *et al.*, 1997; Hass & Longmore, 1979, 1980). In this study we focused on serum lipid-BLES interactions.

Adsorption occurs when there is a propensity for the more hydrophobic regions on the outer surface of a molecule to repel towards air when interacting with the aqueous environment, while the hydrophilic portion is drawn toward the aqueous environment (Yu & Possmayer, 1996). Adsorption studies were conducted to determine the exact mechanism in which BLES surface activity is compromised by serum and its components. Adsorption of BLES and BLES with additive mixtures (**Figure 4**) showed that samples of BLES with serum and LDL had prevented absorption of the BLES film to equilibrium value (~ 25 mN/m). These samples did not reduce γ below 50 mN/m. However, BLES with cholesterol samples were able to reach equilibrium γ in a short time of adsorption, with very little effect compared to BLES alone.

The inhibition by LDL and serum could perhaps be explained by competitive adsorption of specific components of serum or by prevention of the specific BLES lipids to be able to absorb. Since our Raman studies showed that the bilayers of BLES were directly affected by serum and LDL, we would rather assume the latter idea. However, since our AFM studies showed that serum and LDL caused dramatic changes in BLES film structure, it is reasonable to assume some of the additive components could have competitively adsorbed in the in the surface from such bilayers.

Serum derived proteins as well as cellular lipids are normally surface active and can also absorb to the air-water interface, forming films, just like LS. These materials are known to impair LS through specific biophysical interactions as suggested above (Fuchimukai *et al.*, 1987; Holm, 1992; Holm *et al.*, 1999). An earlier study by Holm *et al.*, 1988, showed that albumin and fibrinogen, by way of competitive adsorption,

interfered with the absorption of specific surfactant components to the air-water interface (Holm *et al.*, 1999). The term “competitive adsorption” described serum proteins that formed a film, or portion of film, at the air-water interface and prevented, or delayed, surfactant adsorption by occupying space in the film or the air-water interface and thereby preventing the surfactant aggregates from reaching the interface due to lack of space (Gunasekara *et al.*, 2008; Nag *et al.*, 2010; Zasadzinski *et al.*, 2001, 2005). The results from an *in vitro* study by Holm *et al.*, 1999, had previously suggested such a mechanism. Using Wilhelmy balance and pulsating bubble surfactometer studies to measure adsorption, Holm *et al.*, measured the attenuation of the overall γ with surfactant alone or with the addition of inhibitors. It was found that albumin competed with LS for the air-water interface during adsorption when simultaneously added with the surfactant (Zasadzinski *et al.*, 2005).

The protein inhibitory effects were seen if they reached the surface before occupancy by surfactant molecules. However, albumin could not penetrate if a surfactant film was pre-established and this was the reason the inhibitions could be reversed by excess LS to protein ratios (Reviewed by Holm, 1992; Holm *et al.*, 1999). It is mentioned by Zasadzinski *et al.*, 2005, that a probable cause of adsorption reduction is due to a combination of steric and electrostatic repulsive interactions that are induced by the presence of serum proteins at the air-water interface. They deduced from experiments that serum affected the transport of surfactant to the interface. However, this could be reversed by addition of polymers (Zasadzinski *et al.*, 2005).

Some conflicting results were evident in a study by Gunasekara and colleagues in

2008. They examined two different mechanisms of surfactant inhibition: 1) competition of the air-water interface and 2) impairment of the surfactant film itself. They studied serum proteins, albumin and fibrinogen, in concentrations similar to those in diseased lungs and when additives were added before or after formation of BLES films. In this study, minimal delay or rapid adsorption was noticed in BLES film formation with the presence of a pre-formed protein film. They concluded that surfactant inhibition was likely caused by a dysfunctional film instead of inhibition by way of competitive adsorption of the serum proteins (Gunasekara *et al.*, 2008). This contradicted the previous study by Holm *et al.*, 1999. This may be due to different surfactant concentrations used in the studies as well as insertion of the proteins in the surfactant dispersions by different methods. Different groups have utilized different methods of inserting surfactant inactivators, such as dissolving the additive in the bilayers, or spreading films from solvents with proteins dissolved in the subphase.

Nag *et al.*, 2006, showed that albumin interacted with surfactant lipids in bilayers causing them to form films with a more fluid phase (Nag *et al.*, 2006), demonstrating that inhibition by proteins can occur via interfering with surfactant bilayer structure (Vidyasankar, 2004). Gunasekara *et al.*, noted that although, competitive adsorption was not supported by their study, competitive adsorption might still occur once surfactant has become altered in its composition and unable to keep stable structures and which does not adsorb as efficiently (Gunasekara *et al.*, 2008).

Unlike these proteins, cell membrane lipids and free fatty acids, such as oleic acid, were shown to also readily adsorb and penetrate the films and form mixtures with

lung surfactant lipids (Hall *et al.*, 1992; Holm *et al.*, 1999). Holm *et al.*, 1999, also studied effects of lysophosphatidylcholine (LPC) on lung surfactant. It was noticed that LPC-induced inhibition of LS was due to interactions within the surfactant film during compression. LPC was shown to readily adsorb and penetrate the film and efficiently mix with the film itself (Holm *et al.*, 1999). LPC also had shown a competitive effect with the LS during adsorption. In this study, it was mentioned that due to its conical or ‘wedged’ shape, LPC possibly perturbed the packing of the disaturated phospholipids (DPPC) and thus prevented the low γ values being achieved as in normal surfactant films (Holm *et al.*, 1999; Possmayer *et al.*, 2010). However, a set of recent studies clearly show that excess cholesterol can inactivate various surfactant preparations far more significantly than serum proteins. This was mainly found due to specific molecular interaction of this lipid with either a specific surfactant lipid (DPPC) or its ability to induce major structural changes in the domain morphology of films (Keating *et al.*, 2007, 2011), as well as bilayers (Bernadino *et al.*, 2009; Serna *et al.*, 2004). In our study, a similar effect of minor contaminants like serum, LDL and cholesterol was observed.

Cholesterol and Structural Organization

As mentioned previously, the effect of cholesterol on BLES adsorption was minimal. It has been previously shown that enhancement of adsorption rate of surfactant phospholipids occurs to the equilibrium γ , by physiological levels of cholesterol. This is credited to fluidizing effects of cholesterol on phospholipid mixtures high in DPPC content (Keating *et al.*, 2007; Laing *et al.*, 2009; Silvius, 2003). It was suggested however, that this fluidity of the phospholipid may disrupt such films from

reaching low γ upon compression (Keating *et al.*, 2007). However, Schürch, 1982, showed that normal cholesterol present in the surfactant can lower γ (Schürch, 1982; Gunasekara *et al.*, 2005).

In our study, it was possible that excess cholesterol did not affect the adsorption rate but affected the ability of such surfactant films to reach low γ . This may have been with an increase in fluidity of bilayer dispersions that would help in rapid spreading of surfactant but would also make the monolayer film too fluid to lower γ upon compression as evident from our experimental results. Since a previous study had also shown no difference in the behaviour of surfactant containing various amounts of cholesterol with respect to adsorption (film formation). This study pointed out that both a functional and dysfunctional surfactant are able to reduce the γ to equilibrium, but only a fully functional surfactant can reduce γ to very low values upon compression (Gunasekara *et al.*, 2005). Other studies have shown that excess cholesterol did slow down surfactant adsorption rate (Taneva & Keough, 1997; Keating *et al.*, 2007).

Figure 5 showed the compression-expansion isotherms for BLES and BLES + additive films. As evident from the plots, all materials except 10% cholesterol prevented BLES monolayers from reaching close to 1 mN/m surface tension. As 10% cholesterol films were further compressed, there was indeed the formation of gel type domains but also some others with 'spiky' centers. Also evident was the non-existence of squeeze out for the lipid materials. Somehow serum, cholesterol and LDL interacted with the BLES lipids to prevent complete compression of the monolayer and also interacted with the gel lipids to prevent LS films to reach a low γ . Cholesterol may some

how bind to some of the lipids in the monolayer which in turn prevented the LS monolayer from fully functioning and the LS from reaching a low γ .

Multiple compression-expansion cycles, on BLES (**Figure 5(a)**) showed that the plateau region changed with increasing number of cycles. This suggested that materials from films probably were 'squeezed out' and caused film refining with specific surfactant components as suggested by others (Georke, 1974, 1992; Gunshekara *et al.*, 2004; Nag *et al.*, 1998).

It was suggested that this 'squeeze out' process allowed for the removal of the non-lipid or other fluid phospholipid constituents of the lipid monolayer, thus allowing for enrichment of DPPC in such films. The term 'squeeze out' refers to the removal of material above or below the plane of the air-water interface. As more compression-expansion cycles were performed, the more enriched with DPPC the monolayer became which allowed the surfactant monolayer to achieve low γ values (Yu & Possmayer, 2003). However, recent studies have suggested that the squeeze-out process may not be required as pure fluid-lipid monolayers can be rapidly compressed to lower and close near 0 mN/m γ (Reviewed by Possmayer *et al.*, 2010).

In comparison, (**Figure 5(d)**), BLES with cholesterol (10%) showed different isotherms than those with 20% (**Figure 5(e)**). With BLES with 10% cholesterol (**Figure 5(d)**), the isotherm shown achieved a low γ , which agreed with the fact that the 10% physiological amount of cholesterol naturally found in LS, did not affect the films ability to reach very low γ . The plateau region for BLES with 20% cholesterol (**Figure 5(e)**) was, however different, since there was no significant plateau (squeeze-out) region

observed. This was maybe due to the fact that when excess cholesterol was added, this excess cholesterol could not have been 'squeezed out' as easily as those of other components of LS. The γ_{\min} reached for **(Figure 5(d))** BLES with 10% cholesterol was ~ 5 mN/m, which was almost the same as for BLES alone, and lower than that for BLES with 20% cholesterol **(Figure 5(e))**, which had a minimum γ of ~ 15 mN/m. This trend was also evident in **Figures 5(f) and 5(g)** with the addition of both 10% and 20% LDL to BLES.

These results illustrated that serum and LDL materials mixed well with the BLES lipids in films and could not be easily removed. Clearly a component or several components of these mixtures interacted with BLES lipids to prohibit the surfactant from reaching low γ values and becoming surface active.

This clearly showed that excess cholesterol inhibited surfactant surface activity and made the material somewhat dysfunctional. These results are in agreement to those found in some previous study conducted by Gunasekara *et al.*, 2005, where they used a captive bubble surfactometer (CBS) to investigate the effects of addition of 10% (healthy lung) and 20% (injured lung) by weight of cholesterol to BLES. They found that with 10% cholesterol added to BLES films, a low (close to 1 mN/m) value of γ , similar to normal LS, could have been sustained for a period of time. Samples that contained BLES with 20% cholesterol however did not reach a low γ (Gunasekara *et al.*, 2005).

Cholesterol and LDL are difficult to remove since they are lipids. Cholesterol also has a high affinity for DPPC (Keating *et al.*, 2007). The phospholipid DPPC, containing disaturated chains, can pack tightly in films (and thus produces gel domains) due to these

saturated chains. Cholesterol will normally fluidize these chains or cause packing artifacts and such films would not be expected to reach low γ . However, when present in physiological amounts, they may also rigidify the fluid chains of fluid phospholipids such as 16:0/18:1 (palmitoyl-oleyl-PC) which are also present in significant amounts in BLES (**Figure 3(b)**). This balance of rigidity (ordering) of chains in LS may be overturned by excess cholesterol, mainly since cholesterol is difficult to remove from the lipid environment either by increasing compression or multiple cycling as evident from **Figure 6(a)**.

These effects are far more evident and dramatic for serum and LDL, suggesting possibly cholesterol may not be the only factor in surfactant inhibition. The inability of these serum lipids to be removed would interfere with the tight packing of DPPC lipids as well as DPPC gel domain formation in the surfactant monolayer. ‘Holey’ domains or absence of gel domain structure was evident in a study by Panda *et al.*, 2004, on dysfunctional surfactants which had about twice the amount of normal cholesterol and prevented films from reaching low γ below 20-30 mN/m. Recently, a series of studies have shown that the effects of serum soluble protein on natural surfactant is quite different than those with excess cholesterol or those from dysfunctional lungs (Zuo *et al.*, 2008a-c). Some of the inhibitory effects of excess cholesterol could be removed by either using SP-A or cyclodextrin, a compound which binds and removes cholesterol (Zuo *et al.*, 2008a; Gunaskera *et al.*, 2010). Further studies are required to understand if this removal of inhibition effect can be extended to LDL or whole serum.

By examination of the C_{15} values as seen in **Figure 6**, compressibility of BLES

films was dramatically altered when serum or LDL was added. This may have been due to the competition of these materials with BLES for the formation of the monolayer or some portions of these materials may have somehow bound to BLES molecules and prevented formation of a surface active film. Since serum and LDL contains proteins, it is possible there may be a two-fold mechanism of surfactant inhibition.

Structure and Dysfunction

Atomic Force Microscopy is a useful tool in imaging the structure(s) of biological systems, mostly due to its nano-scale resolution. Various previous studies have used the method to study lipid films as well as those with serum proteins (Devraj, 2005; Zasadzinski *et al.*, 2001; Nag *et al.*, 2002; Nag *et al.*, 2007, 2008; Panda *et al.*, 2004; Parsons, 2005; Vidyasankar, 2004; Zuo *et al.*, 2008a-c). Each study examined effects of serum or serum components on formation of BLES domain formation. It was concluded that serum materials separately or in conjunction with one another prevented BLES domain formation by interacting with the DPPC lipids, or forming their own monolayer film, or sometimes a combination of both.

Deposits of the same samples used in the monolayer studies were taken at γ 52, 42, and 32 mN/m respectively. Prevention of BLES domain formation was seen in all but the 10% cholesterol deposit for 42 mN/m. This was likely do to with the physiological amount of cholesterol that is found in the lung somehow helping to form these domains, such as lipid rafts in cell membranes. Evident from **Figure 8**, serum and LDL were able to form areas of holes and also their own domains. The domains in the holes may have been due to components of serum and LDL preventing surface active

components of LS to adsorb so that they could not form a surface active film. The 'holey' areas may be DPPC remnants or separate domains of serum components either alone or in conjunction with BLES lipids or both (Nag *et al.*, 2007). The 'spiky' area seen in the various samples, especially BLES with 10% cholesterol, could possibly be cholesterol domains, or in other cases, some combination of perturbant and BLES lipid.

Cholesterol when added in excess completely abolished any domain formation. This may have been due to the fact that when cholesterol exceeds the DPPC concentration, the sterol may start to interact with the unsaturated lipids and thus completely keep the monolayer in a fluid state (Keating *et al.*, 2007). In a more recent study by Keating *et al.*, 2010, using mass spectral imaging, it was found that cholesterol caused re-organization of gel phase domains as well as formation of a new liquid-ordered (L_o) phase inside such domain (Keating *et al.*, 2007). They suggested that excess cholesterol interacts with DPPC and reduces the miscibility of other gel-lipids (DPPG) in such domains. Even though we did not observe any L_o phase in our films with excess cholesterol, the holey domains as well as large structures seen in LDL and serum samples (**Figure 9**) could be such L_o phases where the serum proteins or LDL particles may reside.

Previous AFM and fluorescence studies suggested that normally soluble proteins or other bulky perturbants are preferentially adsorbed in the fluid phase of lipid films because of the loose packing of the lipids in that phase (Zasadzinski *et al.*, 2001). This most often resulted in decreased size and amount of tilted condensed domains as addition of the perturbant increased (Nag *et al.*, 2007; Zasadzinski *et al.*, 2001).

An earlier study using fluorescence microscopy showed that high concentrations of serum had caused the formation of smaller BLES domains possibly due to its presence in the fluid phase which inhibited DPPC lipids to segregate into gel domains upon compression. Domains that were probe-excluding were also present. They may have been domains formed by serum components (Nag *et al.*, 2007). In agreement with these findings, an even earlier study showed how injuriously ventilated lungs, similar to diseased lungs, showed increases of serum protein and cholesterol. They also described domain formation in these “diseased” lungs. Very few, if any, DPPC-rich domains were present at high pressure and these domains were noticeably smaller and covered less of the total surface area, despite similar DPPC content (Panda *et al.*, 2004). Injuriouly ventilated surfactant showed almost no gel domains observed in normal surfactant, but instead showed evidence of a larger number of small, intermediate height domains.

Domain height is due to the tilting of the DPPC fatty acid chains in gel phase, more perpendicular with respect to the plane of the monolayer, compared to those in the fluid phase (Diemel *et al.*, 2002; Zasadzinski *et al.*, 2001). These intermediate domains were found to contain areas of some fluidity seen from non-exclusion of fluorescent probes. This was viewed as different types of domain height differences between the two phases in AFM (Nag *et al.*, 2007; Zasadzinski *et al.*, 2001). The higher heights of the gel domains (circular areas) were caused due to tilts of the fatty acyl chains of PLs in gel phase when there was a tighter packing induced by lateral compression of the films as well as due to the molecules’ orientation being more perpendicular to the air-water interface (Nag *et al.*, 2007).

The increase of cholesterol content in this “diseased” surfactant, could have possibly contributed to the alteration of domain formation and because cholesterol causes fluidity in a monolayer LS film, cholesterol or some other components of LDL and serum, may have contributed in some way to the formation of the intermediate domains (Panda *et al.*, 2004). Based on our structural study, prevention of the BLES domains from forming was possibly key in these surfactant films not reaching low γ . Some of the recent studies mentioned in the beginning of the discussion section (Reviewed by Possmayer *et al.*, 2010), suggests that the structural features observed in normal and dysfunctional films require further research. It would be speculative at best to suggest cholesterol is the only component which makes these films dysfunctional, as were have observed structures in LDL and serum containing films which are close to those observed in dysfunctional surfactant films from injured lungs (Keating *et al.*, 2007, 2011; Panda *et al.*, 2004; Zuo *et al.*, 2008c). Although a large number of studies have recently focused on cholesterol, they have not been able to conclusively show how structures in dysfunctional surfactant films contribute directly to the changes in surfactant activity. Even though nano-structured domains, “domains inside domains”, liquid ordered phases, our “holey domains” and spiky domains may somewhat suggest an extremely complex structure-dysfunction relationship, it is the underlying bilayer-monolayer correlation, under dynamic conditions, that may hold the key to understanding surfactant dysfunction (Possmayer *et al.*, 2010).

Bilayer Phase Changes

Raman is a very useful technique to study various organic materials because it is a

non-invasive method. It does not require the use of a probe, intensive sample preparation, as well as its low intensity laser prevents samples from local heating or photodegradation and allowed us to study surfactant bilayers. Recently a number of studies have focused on observing structures in surfactant bilayers using giant unilamellar vesicles (GUV) (Bagatolli, 2006; Bernadino *et al.*, 2009; Reviewed by Possmayer *et al.*, 2010). These bilayer studies suggest that gel-domains found in the normal surfactant bilayers are altered by addition of different components as well as inactivators such as cholesterol. However, a recent study using fluorescent labeled lamellar bodies showed quite dramatically different structures in the surface films formed during adsorption (Ravasio *et al.*, 2010). We did not have the ability to image our LDL and serum containing bilayers but we did observe the general change of fluidity of such bilayers using Raman spectroscopy. Although these domains and subpopulations of lipid species in surfactant bilayers, as observed using fluorescent lipids, may be important in surfactant adsorption, whether such structures truly exist in lamellar bodies is in question, since one study using electron spin resonance failed to detect such organization of lipids in normal surfactant bilayers (Bernadino *et al.*, 2009). However it was pointed out in a recent symposium that the three-dimensional organization of bilayer-monolayer organization is critically dependent on temperature as well as on cholesterol content (Possmayer *et al.*, 2010). Our Raman studies focus on this point mainly.

Figures 9-10 showed an increase in the wavenumber of the ν_s CH₂ peak with an increase in temperature. The shift in wavenumber to higher numbers showed an increase in fluidity of the system. Since the fluidity of BLES bilayers was directly affected by

additives, it is assumed that the films formed from such bilayers would also increase in fluidity. Serum and LDL addition seemed to cause a more fluid bilayer at all temperatures and it was probably due to this fluidity that prevented BLES domains in films, thereby inactivating a functioning surfactant or the lipids ability to be tightly packed, or possibly these gel lipids from reaching the surface.

The bilayer phase changes observed from the order parameter profiles (**Figure 10**) from Raman suggested that LDL and serum alter the fluidity of such membranes, however excess cholesterol does not show any significant effects. It is also puzzling to notice how the cholesterol or its ester in LDL directly affects the BLES bilayers, since the esterified form of cholesterol in LDL (or in serum LDL) cannot directly enter BLES bilayers simply by lipid exchange, since in normal situations these are entered into type II cells by the receptor mediated pathway. Whatever be the case, certain materials from LDL and serum seem to cause fluidity changes of bilayers as detected in the Raman spectroscopy, and this caused possible structure-function changes in the films being adsorbed from such bilayers.

Some previous studies have shown that cholesterol can interact with SP-B and SP-C in LS bilayers which were present in our BLES (Serna *et al.*, 2004). Larsson *et al.*, in a previous paper had suggested that a mechanism by which albumin may inhibit surfactant was by inactivating the SP-B/SP-C from functioning properly (Larsson *et al.*, 2006) to form highly surface active films. In another study, we found cholesterol to slightly increase the order parameter of BLES bilayer in the gel phase using ^2H -NMR and albumin to induce specific fluid domains in such bilayers (Nag *et al.*, 2006). Although

these studies do not suggest any clear cut mechanism of bilayer interactions of LDL and serum with BLES, our Raman data clearly suggests possibilities of different modes of interaction of these serum materials with BLES compared to cholesterol in normal or excess amounts.

It is possible that the apo-proteins present in LDL as well as serum soluble proteins such as albumin and others could have probably interacted with SP-B/SP-C present in BLES and thus caused the changes in BLES bilayers, other than the affects already induced by cholesterol or its esters. There may possibly be a dual mechanism involved here requiring further investigation with serum or LDL protein components.

Conclusions

In summary, excess cholesterol or its esters may actually arrive inside the lung through LDL transport. It was excess cholesterol, or its esters, that was the most potent inactivator of LS since this lipid is more difficult to remove from films. Whole serum, serum proteins and its lipid components may specifically interact with the surfactant films and bilayers by different mechanisms, and each in combination (lipid and protein) may act synergistically. However the complete protein fraction studies (undergoing in our laboratory) would clarify the fact.

LDL, serum and cholesterol altered bilayer packing, surface activity and dramatically altered film structures, indicating some molecular re-arrangements of LS lipids or proteins either in monolayer films or bilayers. However, there seemed to be further inactivator agents in serum and LDL which need to be explained in the future.

Future Studies

In future studies, specific lipid components of serum such as HDL, triglycerides and cholesterol esters should be studied to see their effects on surfactant films and bilayers. One of the major studies requires the specific separation of serum lipid and serum protein fractions and observing their structure-function correlations in inactivating surfactant (Verge, 2010). The specific molecular re-arrangements observed in our study of domains in films need to be further explored in detecting the exact composition of the variety of domain structures using possibly fluorescently labeled LDL, cholesterol and serum proteins, or using mass-spectral imaging.

Currently an investigation is underway in our laboratory on some of these

questions, however, future studies should correlate the true patho-physiological situations in injured lungs and ARDS. This includes sensitive and detailed analysis of increased amounts of the specific lipids and proteins as measured in injured and ARDS lungs, instead of oversimplifying the model by using very high amounts of additives as done over the last four decades. Future investigation into the exact mechanism of biophysical inhibition could allow for how and what actually causes surfactant dysfunction and possibly how such processes can be reversed or help in the treatment of ARDS.

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Appendix A

Publications Arising From This Work

Hiller, A., Fritzen-Garcia, M., Thompson, D., Verge, M., Devraj, R., and Nag, K. (2011). Serum lipids are more potent inhibitor of lung surfactant than serum proteins. *To be submitted*.

Nag, K., Fritzen-Garcia, M., Devraj, R., **Hillier, A.**, and Rose, D. (2008) Phase transition, raft structures and cholesterol in films and bilayers of a natural membranous system. *Structure and Dynamics of Membranous Interfaces*, (Ed. K. Nag), John Wiley & Sons, Hoboken, NJ.

Nag, K., **Hillier, A.**, Parsons, K., Fritzen-Garcia, M. (2007) Interactions of serum with lung surfactant extract in the bronchiolar and alveolar models. *Respiratory. Physiology & Neurobiology*. 157:411-424

Hillier, A., Fritzen Garcia, M., Thompson, D., and Nag, K. (2008) Serum cholesterol and lipoproteins are highly more potent inhibitors of pulmonary surfactant than serum. *INTERNATIONAL CONFERENCE ON LUNG INNATE IMMUNITY AND PULMONOLOGY i-CLIP 2008*, Toronto, P6.

Panda, A.K., **Hillier, A.**, Fritzen Garcia, M., Thompson, D., and Nag, K. (2006) Interactions of serum and its component albumin with lung surfactant membranes. *NATIONAL CONFERENCE ON DISPERSE SYSTEM*, Assam University, Silchar. IL-10B

Hiller, A. (2005) Interactions of serum with lung surfactant in the upper air-way model. (*Hons. Thesis, MUN Library*)



